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MOLECULAR PATHOPHYSIOLOGY UNDERLYING THE NEONATAL FORM OF FARBER DISEASE

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Tese de doutoramento apresentada ao Instituto Ciências Biomédicas Abel Salazar da Universidade do Porto em Ciências Biomédicas

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Tese de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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À minha Mãe...

Legal issues

Preceitos legais

In accord with the 2nd of the 8th article of Decreto-lei nº388/70, part of the results presented in this dissertation were published in the following paper:

De acordo com o n° 2 do Artigo 8° do Decreto-Lei n° 388/70, parte dos resultados apresentados nesta dissertação encontram-se publicados no seguinte artigo:

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The author of this dissertation declares her participation in the outlining and execution of the experimental work, as well as in the interpretation, discussion and drafting of the results.

O autor desta dissertação declara que interveio na concepção e execução do trabalho experimental, assim como na interpretação, discussão e redação dos resultados.

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Abbreviations

AC	Acid ceramidase
AP	Adaptor protein
Arg	Arginine residue
ASAH1	Acid ceramidase human gene
aSMase	Acid sphingomyelinase
Asn	Asparagine residues
ATP	Adenosine tri-phosphate
bp	Base pairs
BMP	Bis(monoacylglycero)phosphate
^{14}C	Radioactive isotope of carbon-14
Ca	Calcium
CD40	Human cluster of differentiation 40
CD63	Human cluster of differentiation 63
CD95	Human cluster of differentiation 95
cDNA	Complementary DNA
Cer	Ceramide
Cer1P	Ceramide-1-phosphate
CERT	Ceramide transfer protein
CLEAR	Coordinated lysosomal expression and regulation
CNS	Central nervous system
COP	Cytoplasmic coat protein
Cys	Cystein residue
del	Deletion
ER	Endoplasmatic reticulum
FD	Farber disease
fs	Frameshift
GalCase	β-Galactocerebrosidase
GalCer	Galactosylceramide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Golgi complex
Glase	β-Galactosidase
Glc	Glucose
GlcCase	β-Glucocerebrosidase

GlcCer	Glucosylceramide
GM1	Ganglioside monosialic 1
GM2	Ganglioside monosialic 2
GM2AP	GM2 activator protein
GSL	Glicosphingolipid
HexT	Total β-hexosaminidase
HMU-PC	6-Hexadecanoylamino-4-methylumbelliferylphosphorylcholine
HRP	Horseradish peroxidase
ins	Insertion
kDa	Kilodalton
LacCer	Lactosylceramide
LAMP	Lysosome associated membrane protein
LE	Late endosomes
LIMP	Lysosomal integral membrane protein
LMP	Lysosomal membrane protein
LSD	Lysosomal storage disease
MIM	Mendelian inheritance in man number
MLII	Mucolipidosis type
mRNA	Messenger ribonucleic acid
mTORC1	Mammalian target of rapamycin complex 1
M6P	Mannose-6-phosphate
M6PR	Mannose-6-phosphate receptor
NPA	Niemann-Pick disease type A
NPB	Niemann-Pick disease type B
NPC	Niemann-Pick disease type C
^{32}P	Radioactive isotope of phosphorus-32
PM	Plasma membrane
Rab	Ras superfamily of monomeric G proteins
RER	Rough endoplasmic reticulum
RT-PCR	Real-time polymerase chain reaction
SAP	Sphingolipid activator proteins
SCARB2	Human scavenger receptor class B, member 2
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Ser	Serine residue
SL	Sphingolipid
SM	Sphingomyelin

SMase	Sphingomyelinase
SMPD1	Sphingomyelin phosphodiesterase 1 human gene
SMS	Sphingomyelin synthase
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SNP	Single-nucleotide polymorphism
Sph	Sphingosine
TFEB	Transcription factor EB
Thr	Threonine residue
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor alpha
UDP	Uridine diphosphate

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Abstract

Farber disease, also known as Farber's lipogranulomatosis (FD, MIM 228000), is a rare autosomal recessive disease caused by mutations in the *ASAH1* gene. This gene codes for acid ceramidase (EC 3.5.1.23), a lysosomal heterodimeric enzyme that is responsible for the hydrolysis of ceramide in sphingosine and fatty acids. FD is, therefore, characterized by the accumulation of ceramide in various cell types. The disease is clinically heterogeneous and 7 subtypes are presently known. Typical symptoms include the unique triad of subcutaneous nodules, painful and progressively deformed joints, and hoarseness by laryngeal involvement. Although 80 patients with the diagnosis of FD are referred in the literature, the genotype was characterized thus far in only 20 patients leading to the identification of 23 mutations in the *ASAH1* gene, mostly missense mutations. Comparing with other lysosomal diseases few causative mutations of FD are known, and for the vast majority of them the molecular mechanism is unclear, thus hampering the establishment of genotype-phenotype correlations. Moreover, the correlation between the clinical phenotype and either the residual acid ceramidase enzymatic activity or the level of ceramide storage is still a controversial issue.

The aim of this thesis was to provide novel insights towards a better understanding of the disease molecular pathogenesis through the investigation of genetic and biochemical basis of the most severe clinical manifestation of acid ceramidase deficiency ever described.

In this study, the genotype of a neonate FD patient with non-immunological hydrops fetalis was elucidated. Direct sequencing of *ASAH1* genomic DNA revealed a causative heterozygous mutation in the donor splice site consensus sequence of intron 11, g.24491A>G (c.917+4A>G), that resulted in the absence of detectable mRNA. Subsequent analysis of *ASAH1* mRNA showed total skipping of exons 3 to 5. Long-range PCR and sequencing led to the identification of a gross deletion, the first one reported in the *ASAH1* gene, g.8728_18197del (c.126-3941_382+1358del) predicting the synthesis of a truncated polypeptide, p.Tyr42_Leu127delinsArgfs*10. Accordingly, no molecular forms corresponding to precursor or proteolytically processed mature protein were observed. Molecular findings in the non-consanguineous parents confirmed the compound heterozygous *ASAH1* genotype identified in the patient. The identification of two heterozygous loss-of-function mutations provided experimental evidence for the absence of any functionally active acid ceramidase in patient's fibroblasts, underscoring the severity of the clinical phenotype. This data also suggests screening for gross deletions in

other patients in whom the mutation present in the second allele had not yet been identified to elucidate further its overall contribution for the molecular pathogenesis of this devastating disease.

As data from molecular studies support the existence of a deep blockage of the last step of the sphingolipid degradation pathway in patient's cells, the intracellular level of two structurally close and metabolically linked shingolipid molecules, ceramide and sphingomyelin, was investigated. The total level of sphingomyelin *vs.* ceramide was found significantly decreased in FD cells, but an analogous disproportion, i.e. a significant increase, was not seen in Niemann Pick-type B cells that are characterized by the accumulation of sphingomyelin due to the deficient enzymatic activity of acid sphingomyelinase. This observation suggests that the imbalance of total sphingomyelin to total ceramide observed in FD fibroblasts is likely to be due not only to the abnormally high level of ceramide but also to a slightly decreased level of sphingomyelin. The steady state cell level of these metabolites results from a complex network of enzymatic reactions catalysed by several ceramidases and sphingomyelinases. If acid sphingomyelinase, whose enzymatic activity was found particularly decreased in fibroblasts of the neonatal FD patient, represents a disease biomarker is an issue that warrants further investigation. Altogether, data here reported suggest that secondary imbalances such as the residual acid sphingomyelinase enzymatic activity and the cellular content and distribution of sphingolipids might have clinical relevance and also strengthen the hypothesis of an *in vivo* interaction between acid ceramidase and acid sphingomyelinase.

Overall, the data described in this thesis made two contributions in this particular field of the scientific knowledge: detailed analysis on the biological consequences of novel *ASAH1* mutations provided a better understanding of the primary defect of the neonatal FD; enzymatic and biochemical sphingolipid data analysis revealed secondary defects whose implication for the disease pathophysiology must continue to be investigated in the future.

Key-words: Lysosomal disease, Farber disease, Acid ceramidase, *ASAH1* gene, Gross deletion, Splicing mutation, Ceramide, Sphingomyelin.

Resumo

A doença de Farber (Lipogranulomatose de Farber, MIM 228000) é uma doença rara, de transmissão autossómica recessiva, causada por mutações no gene *ASAH1*. Este gene codifica para a ceramidase ácida (EC 3.5.1.23) que é uma enzima heterodimérica responsável pela hidrólise da ceramida em esfingosina e ácido gordo. A doença de Farber é, por isso, caracterizada pela acumulação lisossomal de ceramida em vários tipos de células. Esta patologia é clinicamente heterogénea, estando descritos 7 subtipos. Os sintomas clínicos típicos incluem a presença de nódulos subcutâneos, a deformação progressiva das articulações e rouquidão causadas por alterações na laringe. Dos 80 doentes de Farber referidos na literatura, apenas 20 foram caracterizados genotipicamente o que originou a identificação de 23 mutações no gene *ASAH1*, a maioria das quais mutações *missense*. Comparativamente com outras doenças lisossomais, o número de mutações causais é reduzido e para a maioria delas o mecanismo molecular não está bem estabelecido dificultando, assim, o estabelecimento de correlações genótipo-fenótipo. Além disso, a correlação entre o fenótipo clínico e a atividade enzimática residual da ceramidase ácida ou os níveis de acumulação de ceramida é ainda uma questão controversa.

Este projeto foi desenvolvido com o objetivo de promover o conhecimento sobre a patogénese molecular desta doença através da investigação das bases genéticas e bioquímicas da forma clinica mais grave da deficiência em ceramidase ácida até hoje descrita.

Neste trabalho, o genótipo de um doente de Farber com a forma neonatal e apresentando hidropsia fetal não imune foi elucidado. A análise do DNA genómico do gene *ASAH1* revelou a presença em heterozigotia da mutação g.24491A>G (c.917+4A>G), no local doador de *splicing* do intão 11, resultando na ausência de mRNA detectável. A análise subsequente do mRNA/*ASAH1* revelou a excisão da totalidade dos exões 3 a 5. A conjugação das técnicas *Longe-range PCR* e sequenciação permitiram a identificação da primeira grande deleção no gene *ASAH1*, 8728_18197del (c.126-3941_382+1358del), sugerindo a síntese de um polipéptido truncado, p.Tyr42_Leu127delinsArgfs*10. Estudos ao nível da proteína revelaram ausência das formas moleculares precursora ou madura da enzima. O estudo molecular dos pais não consanguíneos confirmou o genótipo heterozigótico do doente. Assim, a identificação e caracterização destas duas mutações heterozigóticas evidenciou a inexistência de ceramidase ácida funcional em fibroblastos deste doente corroborando, assim, a

severidade do fenótipo clínico. Estes resultados também demonstram a importância do rastreio de grandes deleções em doentes compostos heterozigóticos e cuja mutação no segundo alelo ainda não tenha sido identificada, já que este tipo de mutação poderá estar implicado na patogénese molecular da doença.

Tendo em conta que os resultados obtidos sugerem um bloqueio profundo do último passo da degradação dos esfingolípidos nas células do doente com a forma neonatal de Farber, os níveis intracelulares de duas moléculas esfingolípicas, estrutural e metabolicamente relacionadas, a ceramida e a esfingomielina, foram investigados. Em fibroblastos do doente foi observada uma redução significativa do nível total de esfingomielina *vs.* ceramida em relação a células controlo, mas uma desproporção análoga, isto é, um aumento significativo, não foi observado em células de doentes Niemann Pick-tipo B (células caracterizadas pela acumulação de esfingomielina devido à deficiente atividade enzimática da esfingomielinase ácida). Esta observação sugere que o desequilíbrio entre os níveis de esfingomielina total e ceramida total obtidos em fibroblastos do doente deverá resultar não só de um nível anormalmente elevado de ceramida mas também de um nível ligeiramente diminuído de esfingomielina. Na células, o nível de equilíbrio destes metabolitos resulta de uma rede complexa de reações enzimáticas catalisadas por diversas ceramidases e esfingomielinases. Uma vez que nos fibroblastos do doente de Farber foi observada uma redução significativa da atividade enzimática da esfingomielinase ácida, a possibilidade desta enzima representar um biomarcador da doença é uma questão que deverá ser investigada no futuro. Globalmente, os resultados obtidos no âmbito do presente trabalho sugerem que alterações secundárias, tais como a atividade enzimática residual da esfingomielinase ácida, ou o conteúdo e distribuição celulares dos esfingolípidos, poderão ter relevância clínica e, adicionalmente, apoiam a hipótese de uma interação *in vivo* entre a ceramidase ácida e a esfingomielinase ácida.

Em suma, os resultados descritos nesta tese proporcionaram duas contribuições neste domínio específico do conhecimento científico: a análise detalhada sobre as consequências biológicas das mutações novas identificadas no gene *ASAH1* proporcionou uma melhor compreensão do defeito primário da forma neonatal da doença de Farber; a análise enzimática e bioquímica dos esfingolípidos revelou defeitos secundários cuja implicação para a fisiopatologia da doença deve continuar a ser investigada no futuro.

Palavras-chave: Doenças lisossomais, Doença de Farber, Ceramidase ácida, Gene *ASAH1*, Grande deleção, Mutação de *splicing*, Ceramida e Esfingomielina.

Chapter I

Introduction

I.1. LYSOSOME

In 1949, Christian de Duve, then chairman of the Laboratory of Physiological Chemistry at the University of Louvain in Belgium, was studying how insulin acted on liver cells. He wanted to determine the location of glucose-6-phosphatase, an important enzyme in regulating blood sugar levels. He and his group obtained cellular extracts by blending rat liver fragments in distilled water and centrifuging the mixture at high speeds. They observed high phosphatase activity in the extracts. However, when they tried to purify the enzyme from cellular extracts, they could precipitate the enzyme, but they could not redissolve it. Instead of using cellular extracts, they decided to use a more gentle technique that fractionated the cells with differential centrifugation, separating different components of cells based on their sizes and densities. The researchers ruptured the rat liver cells and then fractionated the samples in a sucrose medium. They succeeded in detecting the enzyme's activity in what is known as the microsomal fraction of the cell.

As control for their experiments, the scientists were using the enzyme acid phosphatase. To their surprise, the acid phosphatase activity after differential centrifugation was only 10% of the expected enzymatic activity (i.e., the activity they obtained in their previous experiments using cellular extracts). One day, by chance, a scientist purified some cell fractions and then left them in the fridge. Five days later, after returning to measure the enzymatic activity of the fractions, they observed the enzymatic activity levels they were looking for. To ensure there was no mistake, they repeated the experiment a number of times. Each time, the results were the same: if they measured the enzymatic activity using fresh samples, then the activity was only 10% of the activity obtained when they let the samples rest for five days in the fridge. To explain these results, they hypothesized that a membrane-like barrier limited the accessibility of the enzyme to its substrate. Letting the samples rest for a few days gave the enzymes time to diffuse. They described the membrane-like barrier as a "saclike structure surrounded by a membrane and containing acid phosphatase". By 1955, additional hydrolases were discovered in these saclike structures (de Duve *et al.,* 1955), suggesting that they were a new type of organelle with a lytic function. De Duve named these new organelles "lysosomes" to reflect their lytic nature. That same year, an experienced microscopist, Novikoff from University of Vermont, was able to obtain the first electron micrographs of the new organelle from samples of partially purified lysosomes. Using a staining method for acid phosphatase, de Duve and Novikoff confirmed its location in the lysosome using light and electron microscopy studies (Essner and Novikoff, 1961), but their functional

significance was only understood later after biochemical studies (rev. in De Duve and Wattiaux, 1966).

For long time, we know that lysosomes contain hydrolases that are capable of digesting all kinds of macromolecules. Christian de Duve, Albert Claude, and George Palade were recognized for their work detailing the functions of the lysosome, when they were awarded the Nobel Prize in Physiology or Medicine in 1974. Nowadays, new roles of lysosomes are emerging and their implication in health and disease is next discussed.

I.1.1. Ultrastructure and function

Lysosomes are found in almost all eukaryotic cells. They are intracellular organelles of about 0.5 μm diameter, enclosed by a membrane, and considered for a long time the organelle involved in the degradation and recycling of cellular waste (rev. in Bärrett and Heath, 1977).

Various methods have been used to purify lysosomes and analyze their proteome. Some of these approaches are based on subcellular fractionations or on the specific feature of soluble lysosomal proteins, e.g. the presence of the mannose-6-phosphate (M6P) modification of their carbohydrate chains. On the basis of present data about 110 lysosome-resident proteins have been identified: ~65 are matrix proteins and ~45 are lysosomal membrane proteins (LMPs). The most abundant LMPs are lysosome associated membrane proteins 1 and 2 (LAMP1 and LAMP2) and lysosomal integral membrane protein 2 (LIMP2, also known as SCARB2) and the tetraspanin CD63 (rev. in Schröder *et al.,* 2010).

The soluble hydrolases, which are active at acidic pH, include proteases, nucleases, glycosidases, lipases, phosphatases and sulfatases that target specific substrates for degradation into their structural monomers (amino acids, fatty acids, simple sugars and nucleotides) and reutilization by the cell. Their collective action is responsible for the total catabolic ability of the lysosome.

Like all other intracellular organelles, the lysosome also has a unique surrounding membrane, with an H^+ pump (Figure 1.1) that uses the energy of ATP hydrolysis to translocate H^+ into the lysosome, thereby maintaining the lumen at its acidic pH (5.0), necessary for optimal activity of lysosomal enzymes. Most of the LMPs are unusually highly glycosylated which helps to protect them from the luminal lysosomal proteases. In

this way, the contents of the cytosol are doubly protected against attack by the cell´s own digestive system. Even if the lysosomal membrane leaks out, digestive enzymes can do little damage at the cytosolic pH of about 7.2. Besides the lysosomal membrane protection function and the transport of metabolites into and out of lysosomes, LMPs are also involved in fusion events of the lysosomal membrane with other cell structures, such as late endosomes (LE), autophagosomes and the plasma membrane (PM) (rev. in Eskelinen *et al.*, 2003).

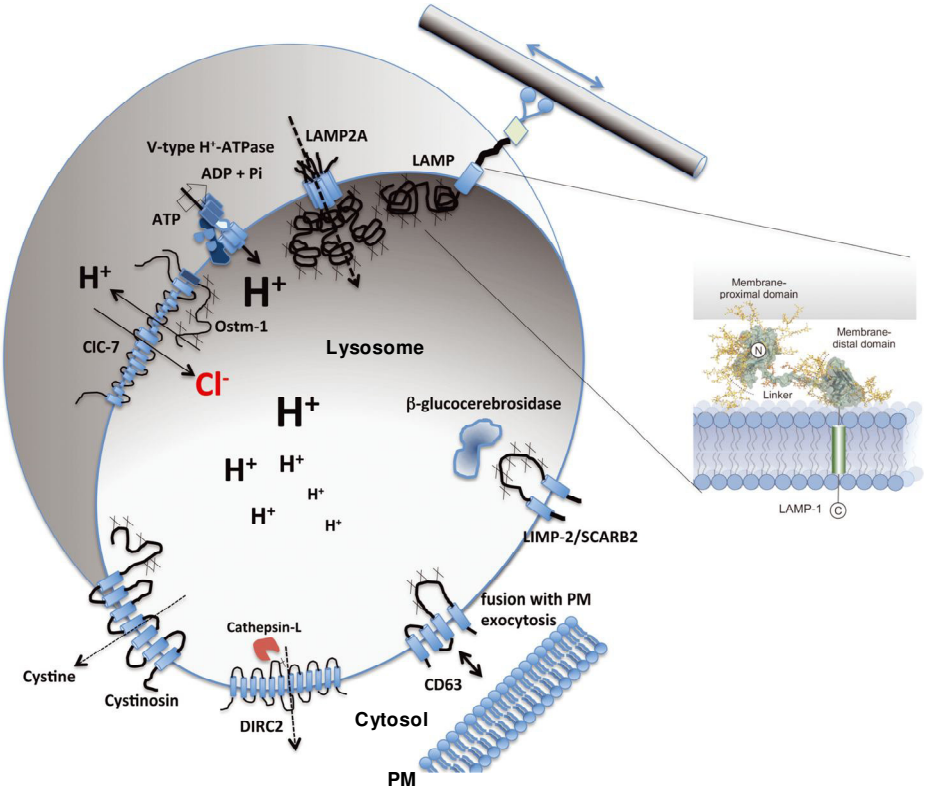

Figure 1.1. Major components of lysosomal membrane. The multi-subunit v-type H^+ ATPase mediates transport of protons into the lysosomal lumen and the chloride channel ClC-7 drives chloride anion transport into the lysosome using the proton gradient. CD63, human cluster of differentiation 63; DIRC2, disrupted in renal carcinoma 2; LAMP, lysosome-associated membrane proteins; SCARB2, human scavenger receptor class B, member 2. Figure taken from rev. in Schwake *et al.,* 2013.

On the basis of current knowledge, three general main functions can be attributed to lysosomes: degradation, secretion and signaling.

As a catabolic cell compartment, hydrolases ensure the degradation of a wide variety of intracellular and extracellular molecules that reach lysosomes using specific cell routes.

Lysosomal secretion occurs through a process called lysosomal exocytosis. In this process, lysosomes fuse with the PM through a Ca^{2+}-regulated mechanism (rev. in Micaroni, 2012). Lysosomal exocytosis is transcriptionally regulated by the transcription factor EB (TFEB) which induces both the docking and fusion of lysosomes with the PM by modulating the expression of specific genes (Medina *et al.,* 2011). Lysosomal exocytosis has been implicatef in several physiological processes, such as microorganism defense mechanism, bone resorption by osteoclasts, melanocyte function in pigmentation, platelet function in coagulation, hydrolase release by spermatozoa during fertilization and PM repair (rev. in Settembre *et al.,* 2013).

Lysosomal signaling refers to its role in nutrient sensing and, subsequently, in a signaling pathway(s) that is involved in cell metabolism and growth, keeping the balance between biosynthetic and catabolic cell states (rev. Zoncu *et al.,* 2011). This function involves the kinase complex mammalian target of rapamycin complex (mTORC1). Growth factors, hormones, amino acids, glucose (Glc), oxygen and stress are the major activators of mTORC1, which in turn positively regulates lipid biosynthesis and ATP production (rev. in Laplante and Sabatini, 2012). The recent observation that the level of mTORC1 exerts its activity on the lysosomal surface (Sancak *et al.,* 2010) and that the amino acids must accumulate in the lysosomal lumen in order for mTORC1 to bind and become activated (rev. Zoncu *et al.,* 2011), support the idea that mTORC1 activity is dependent on lysosome.

In spite of the progresses described above, little is known about how lysosomal function varies in different cells, tissues, life stages and individuals, as well as under distinct physiological conditions. However, to fulfill these varied roles, lysosomes must be present at different levels in different tissues and cell types and their makeup within these different tissues diversify. The complexity of this organelle is reflected by its highly polymorphic ultrastructure, its spatial and functional affinity with endocytic compartments and its temporal continuation with the endoplasmic reticulum and secretory vesicles. Therefore, the biogenesis of this organelle is a complex process requiring the coordinated expression of many soluble and membrane proteins.

I.1.2. Biogenesis

Lysosome biogenesis involves the integration of components of the biosynthetic, exocytic and endocytic pathways of the cell.

I.1.2.1. Biosynthetic pathway

Lysosomal proteins are mostly glycoproteins consisting of mannose-rich oligosaccharides. The biosynthesis occurs on ribosomes associated with the rough endoplasmic reticulum (RER), with subsequent translocation of the polypeptides into the lumen of RER, where specific asparagine residues (Asn-X-Ser/Thr) are N-glycosylated. Two pathways are known to target proteins to lysosomes: the M6P dependent pathway and the M6P independent pathway (rev. in Kornfeld and Mellman, 1989).

During its vectorial transport through the Golgi complex (GC), glycoproteins are proteolytic and oligosaccharide processed. Mannose-rich N-oligosaccharides are converted into complex N-oligosaccharides or hybrid structures, and the phosphate group is added by the action of the enzymes N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase and uridine diphosphate (UDP)-N-acetylglucosamine-1-phosphotransferase, with formation of the sorting lysosomal marker M6P. In the *trans*-GC, lysosomal glycoproteins bind to specific membrane receptors, M6P receptors (M6PR): cation-independent M6PR and cation-dependent M6PR. These receptors recognize M6P residues and are responsible for lysosomal vesicular transport of glycoproteins from the *trans*-GC to the LE. Thus, segregation among the lysosomal protein and protein secretion occurs in the *trans*-GC (rev. in Luzio *et al*. 2000; rev. in Mullins and Bonifacino, 2001).

In LE and hybrid structures, decreased pH (5-6) causes the separation between the receptor and the lysosomal protein. The lysosomal enzyme is dephosphorylated to prevent its return to the GC and the M6PR are included in membrane vesicles which transport it back to the GC or to the PM, for reutilization (Figure 1.2). Thus, lysosomes are defined not only by the presence of acid hydrolases and integral membrane glycoproteins but also by the absence of the two M6PR and recycling cell surface receptors, which distinguishes them from endosomes (rev. in Luzio *et al*. 2000; rev. in Mullins and Bonifacino, 2001).

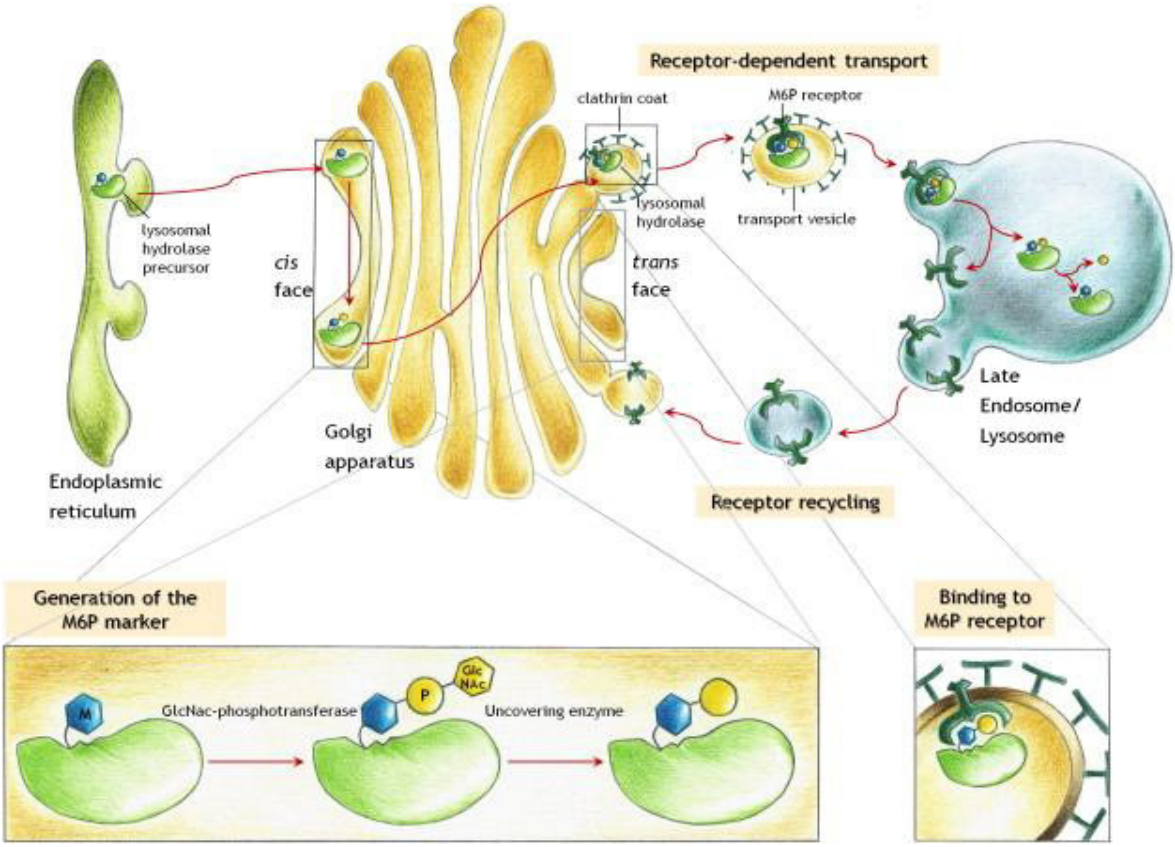

Figure 1.2. Sorting and targeting of lysosomal proteins by M6P receptors. Lysosomal hydrolase precursors are synthesized in the endoplasmatic reticulim, then modified and sorted in the Golgi apparatus. The lysosomal hydrolases are modified by GlcNAc phosphotransferase, which adds M6P to these proteins. These modified lysosomal hydrolases are bound by M6P receptors in the Golgi apparatus. The receptor-protein complexes are transported in vesicles to the late endosomes. Due to the low pH in endosomes, the M6P receptor-protein complexes dissociate. The lysosomal hydrolases remain in the endosomes and the endosomes mature into lysosomes. The M6P receptors are recycled back to the Golgi apparatus via vesicle transport. GlcNAc, acetylglucosamine; M, mannose residue; M6P, mannos-6-phospate; P, phospate. Figure taken from rev. in Coutinho *et al*., 2012.

Although M6PR play a major role in the intracellular transport of newly synthesized lysosomal enzymes in mammalian cells, several lines of evidence suggest the existence of an alternative mechanism of lysosomal targeting (rev. in Ni *et al.,* 2006). LIMP2 was implicated in the delivery of β-glucocerebrosidase (GlcCase), the defective enzyme in patients with Gaucher disease to the lysosomes (Reczek *et al.,* 2007). Sortilin is a multifunctional receptor capable of binding different ligands, including neurotensin (Munck *et al.,* 1999), and has been suggested to mediate GC-to-lysosome transport of the sphingolipid activator proteins (SAPs) prosaposin and GM2 activator protein (GM2AP, Lefrancois *et al.,* 2003; Hassan *et al.,* 2004), acid sphingomyelinase (aSMase, Ni and Morales, 2006) and cathepsins D and H (Canuel *et al.,* 2008).

I.1.2.2. Exocytic pathway

The exocytic pathway moves cargo from the endoplasmatic reticulum (ER) through the GC to the PM by vesicular transport pathways in a succession of vesicle budding and fusion reactions (Figure 1.3). Vesicle coat proteins drive the formation of these vesicles: cytoplasmic coat protein (COP) II buds vesicles from ER to *cis*-GC and clathrin/adaptor protein 1 (AP1) is involved in budding from the *trans*-GC to LE (rev. in Bonifacino and Glick, 2004; Lee and Goldberg, 2010). Cargo-packed vesicles (clathrin/AP1 and clathrin/AP2) formed at the *trans*-GC fuse with the PM to deliver PM resident proteins and secreted proteins (Tokarev *et al.,* 2009). In addition, resident proteins that move to the next compartment have to be retrieved back to the original compartment for maintenance of compartment identity. Therefore, for every step of forward transport, there is a corresponding retrograde transport step. The two major intersections of this bi-directional trafficking are the ER-GC intermediate compartment (ERGIC), which recycles proteins back to the ER mediated by COP I, and recycling endosomes, which recycle proteins back to the PM or the GC (rev. in Saraste and Goud, 2007).

I.1.2.3. Endocytic pathway

Endocytosis is a vital process for mammalian cells by which they communicate with their environment, internalize nutrients, hormones, or growth factors, or take up extracellular fluids and particles. Endocytic mechanisms include phagocytosis, macropinocytosis, clathrin-mediated endocytosis or caveolin-mediated endocytosis, according to the nature of the cargo. The clathrin/AP2-mediated endocytosis (Figure 1.3.) is the best studied one (rev. in Rodemer and Haucke, 2008).

The degradative endocytic pathway starts at the PM, with the involvement and fusing of the cargo with PM and the formation of endossomal vesicles, and ends in lysosomes. Between these two ‘stations’, endocytosed cargo passes through a range of endosomal intermediates that are distinguished by their content, molecular composition, morphology and pH and by the kinetics by which endocytic tracers reach them (rev. in Sachse *et al.,* 2002).

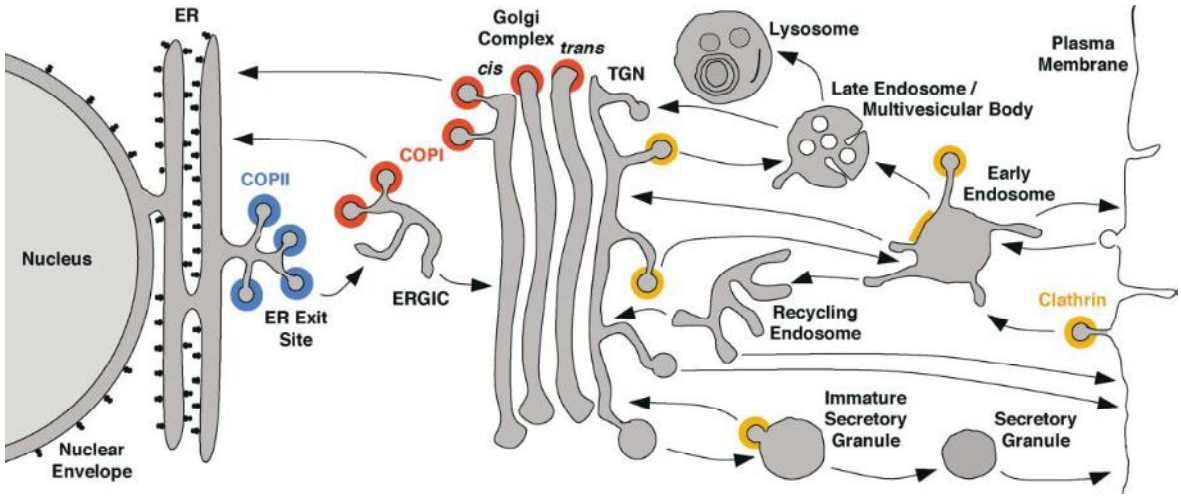

Figure 1.3. Vesicle coat proteins-mediated exocytic and endocytic pathways. COPII (blue) forms vesicles for anterograde transport from the ER to the GC, whereas COPI (red) forms vesicles for both intra-GC transport and retrograde transport from the GC to the ER. Clathrin (yellow) forms multiple complexes based on its association with different APs. Clathrin that is associated with AP1 and AP3 forms vesicles for transport from the trans-GC to the LE compartments, and also for transport that emanates from the early endosomal compartments. Clathrin that is associated with AP2 forms vesicles from the plasma membrane that transport cargo to the early endosomes. AP, adaptor protein; COP, coat protein; GC, Golgi complex; ER, endoplasmatic reticulum; LE, late endosome. Figure taken from Bonifacino and Glick, 2004.

The mechanism of transfer of endocytosed material from endosomes to lysosomes remained controversial, with several theories being proposed. These included maturation (of the endosome into a lysosome), vesicular transport (with vesicles carrying cargo from endosomes to lysosomes), kiss-and-run (a continuous cycle of transient contacts or 'kisses' between endosomes and lysosomes, during which material is transferred between the organelles and each contact is followed by a dissociation or 'run'), direct fusion (of the endosome to the lysosome to form a hybrid organelle) and fusion–fission (a variation of direct fusion and kiss-and-run, in which lysosomes re-form from hybrid organelles as a result of fission events) (rev. in Luzio *et al.,* 2003; rev. in Luzio *et al.,* 2007b). Time-lapse confocal microscopy experiments have shown that kissing and direct fusion events both contribute to the mixing of the contents of endosomes and lysosomes in living cells (Bright *et al.,* 2005). Endosomal trafficking and fusion are mediated by specific sets of membrane-associated Rab GTPases (rev. in Zerial *et al.,* 2001) and SNARE proteins (rev. in Jahn and Scheller, 2006). The fusion of endosomes with lysosomes creates hybrid organelles in which the bulk of the endocytosed cargo is degraded. Reformation of lysosomes from these hybrid organelles requires retrieval and/or recycling of some membrane proteins by vesicular traffic (Bright *et al.,* 2005; Pryor *et al.,* 2005). This view of lysosomes as fusogenic organelles is consistent with other data showing that lysosomes can also fuse with phagosomes, autophagosomes and the PM under appropriate

circumstances (Luzio *et al.,* 2007a, 2007b). Under physiological conditions, endolysosomes and autolysosomes are transient organelles.

The biogenesis and functioning of endosomal and autophagosomal pathways is controlled by TFEB, which regulates the expression of 471 genes that constitute the CLEAR (coordinated lysosomal expression and regulation) gene network (Sardiello *et al.,* 2009; Palmieri *et al.,* 2011). Recent work indicates that non-active TFEB is highly phosphorylated and associates with LE/lysosomes (Roczniak-Fergunson *et al.,* 2012). Autophagy-inducing conditions (e.g., deprivation of Glc or amino acids) result in reduced TFEB phosphorylation, leading to its translation into the nucleus (Peña-Llopis and Brugarolas, 2011; Peña-Llopis *et al.,* 2011) and transcriptional expression of CLEAR genes (Palmieri *et al.,* 2011). Interestingly, TFEB can interact with mTORC1 and associated proteins (Settembre *et al.,* 2012; Martina and Puertollano, 2013), suggesting a mechanism by which the lysosome regulates its own biogenesis by controlling TFEB subcellular localization.

I.2. SPHINGOLIPIDS

I.2.1. Structure and function

Sphingolipids (SLs) are a complex class of molecules that are found in essentially all animals, plants and fungi, and some prokaryotic organisms and viruses. Structurally, SLs are amphipathic molecules that have both hydrophobic and hydrophilic properties. They consist of a sphingoid long chain base (normally sphingosine (Sph), sphinganine or phytosphingosine) to which a fatty acid is attached by an amide bond to carbon 2 (hydrophobic region) and a headgroup at the primary hydroxyl (hydrophilic region). The headgroups range in complexity from a simple H in ceramide (Cer) to complex SLs, including sphingomyelin (SM) and glycosphingolipids (GSLs) with a few to dozens of sugar residues. Thus, Cer constitute the hydrophobic backbone of all complex SLs (Figure 1.4.). The fatty acid chain length of Cer can vary from 2 to 28 carbons, but C-16 to C-24 Cer are the most abundant in mammalian cells (rev. in Merrill *et al.,* 2007).

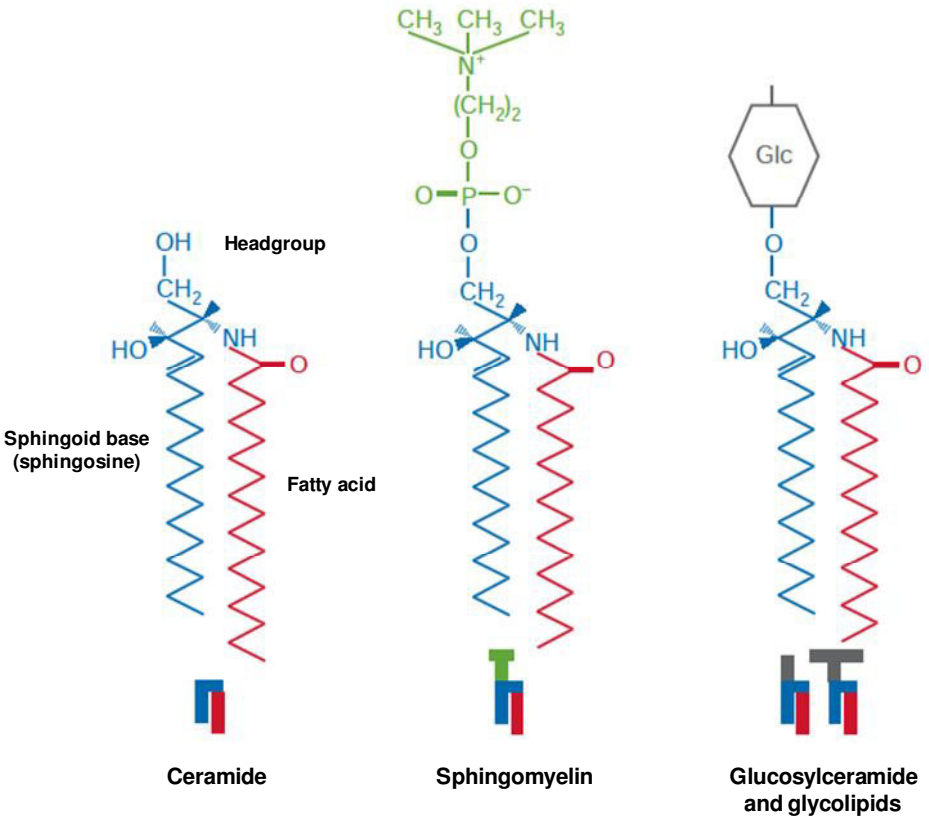

Figure 1.4. Structure of sphingolipids. Ceramide is the simplest SL and the backbone of all complex SLs. Note that, for simplicity, only one kind of sphingoid base (sphingosine in blue), is shown to which only one kind of fatty acid (palmitic acid, in red) is N-acylated. Similarly, only one GSL, (with one Glc residue in grey) is shown, but sequential addition of other carbohydrate residues results in >500 known GSL structures. Glc, glucose; GSL, glycosphingolipid; SL, sphingolipid. Figure adapted from Futerman and Hannun, 2004.

Together with phospholipids and cholesterol, SM and GSLs are characteristic components of cellular membranes. They are predominantly found in the PM, and to a lesser extent in intracellular membranes (rev. in van Meer and Hoetzl, 2010). Besides their structural role, SLs have also been implicated as cell-signaling modulators in a variety of vital cellular processes like differentiation, migration, apoptosis, cell proliferation, cell cycle arrest, senescence, and inflammation. They are located in multiple intracellular organelles, including GC membranes, mitochondria and nucleus, and intracellular vesicles (rev. in Dickson, 2008). In some cases, they are organized in small, dynamic aggregates within membranes referred to as ‘‘rafts’’ where they can influence the localization and functions of transporters and receptors (Hou *et al.,* 2008).

I.2.2. Metabolism and transport

SLs are synthesized in the ER from non-sphingolipid precursors. Despite the structural diversity, their synthesis and degradation are conducted by common anabolic and catabolic pathways. The synthesis of Cer, common to all SLs, can occur through three major pathways: *de novo* biosynthesis, SM hydrolysis, and the salvage pathway (Figure 1.5.).

I.2.2.1. *De novo* biosynthesis

The *de novo* biosynthesis of Cer occurs at the cytosolic leaflet of the ER (Hirschberg *et al.,* 1993; Michel *et al.,* 1997) and begins with condensation of serine and palmitoyl-coenzyme A by serine palmitoyltransferase to form 3-ketosphinganine. In the next steps, the 3-ketosphinganine is reduced to sphinganine by 3-ketosphinganine reductase (Mandon *et al.,* 1992) followed by N-acylated to dihydroceramide by dihydroceramide synthase (rev. in Pewzner-Jung and Futerman, 2006). The resultant dihydroCer is converted to Cer via insertion of a 4,5-transdouble bond into the sphingoid base backbone by dihydroCer desaturases (Michel *et al.,* 1997). Cer is then trafficked to GC via vesicular transport, coatomer protein dependent (rev. in Watson and Stephens, 2005) or via the Cer transfer protein (CERT) (Hanada *et al.,* 2003) where it is the substrate for the synthesis of more complex SLs, including SM and glucosylceramide (GlcCer) which is, then, converted into hundreds of complex GSLs. Synthesis of SM in the GC (Futerman *et al.,* 1990; Jeckel *et al.,* 1990) is catalyzed by two SM synthases, SMS1 and SMS2 (Voelker and Kennedy, 1982). Cer is also metabolized to SM in the PM (Malgat *et al.,* 1986; van den Hill *et al.,* 1985) by the action of SMS2 (Huitema *et al.,* 2004).

Most of complex GSLs are synthesized by the stepwise addition of carbohydrate molecules. Galactosylceramide (GalCer) and GlcCer, are synthesized using UDP-GlcCer glucosyltransferase and UDP-GalCer galactosyltransferase, respectively. Synthesis of GalCer occurs in the lumen of the ER whereas GlcCer is thought to be made on the cytosolic surface of GC (Sprong *et al.,* 2003). GlcCer is followed by the addition of galactose to form Galβ1-4Glcβ1Cer (lactosylceramide, LacCer), which provides the branch point for the synthesis of all GSL series: ganglio (galNAcβ1-4gal), globo (galα1-4gal), isoglobo (galα1-3gal) lacto (galβ1-3glcNAcβ1-3gal), and neolacto (galβ1-4glcNAcβ1-3gal). Thus, UDP-GlcCer glucosyltransferase, which generates the Cer monohexoside precursor of LacCer, is a major control point for the regulation of GSLs

biosynthesis (Lingwood, 2011; rev. in Merrill, 2011; rev. in Yu *et al.,* 2004, rev. in Zeng and Yu, 2008).

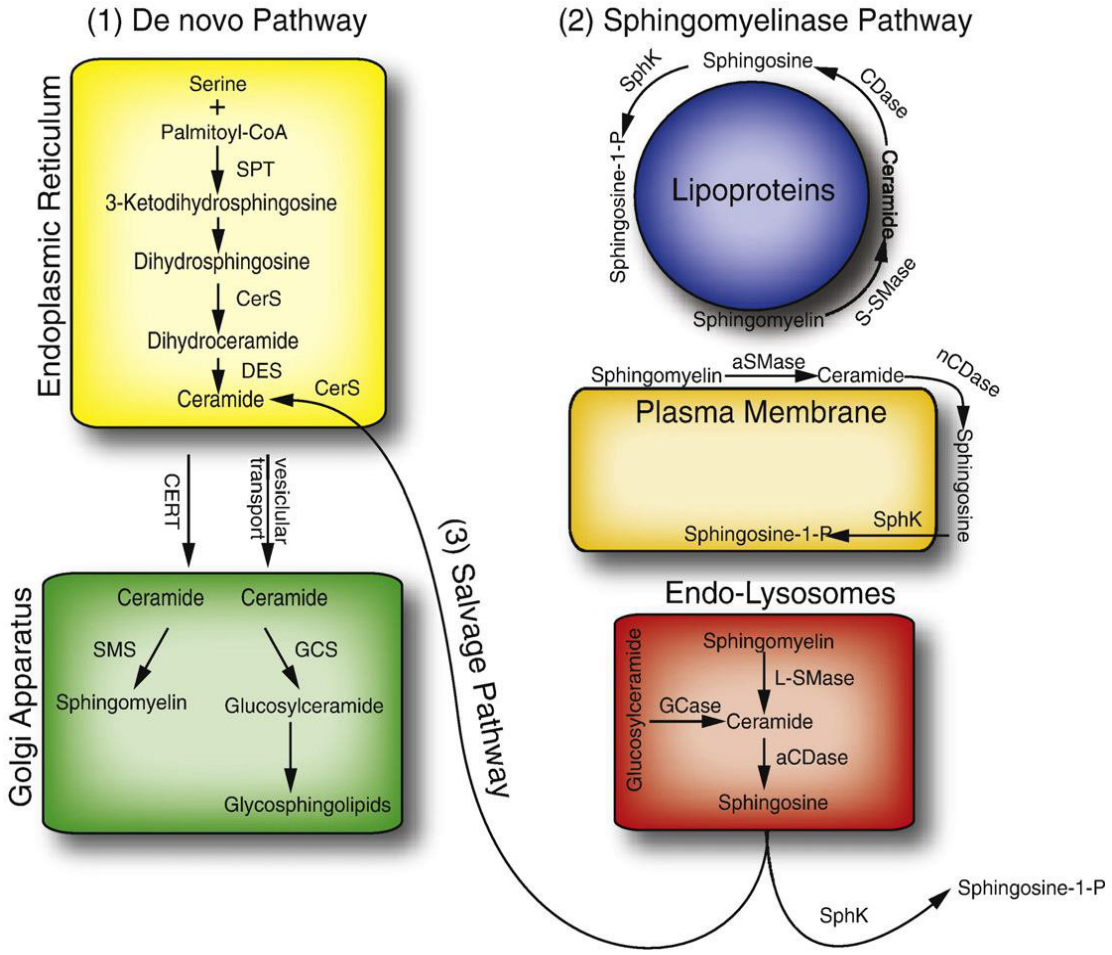

Figure 1.5. Sphingolipids metabolism. (1) *De novo* synthesis of SLs occurs in the endoplasmatic reticulum (ER). Ceramide (Cer) formed in this compartment is transported to the Golgi complex (GC), which is the site of synthesis of sphingomyelin (SM) and glucosylceramide (GlcCer), with the latter serving as the precursor for complex SLs (glicosphingolipids, GLSs). The transport of Cer to the GC occurs either through the action of the transfer protein CERT, which specifically delivers Cer for SM synthesis, or through vesicular transport, which delivers Cer for the synthesis of GlcCer. Delivery of SM and GSLs to the plasma membrane (PM) appears to occur by vesicular transport. **(2)** Sphingomyelinase pathway: The breakdown of sphingomyelin by aSMase occurs within the endo-lysosomal system, at the outer leaflet of the PM, and in association with lipoproteins. In endo-lysosomal system, SM and GlcCer are degraded by the actions of sphingomielinases (SMases) and glucosidases (GCase) to form Cer. By the action of acid or neutral ceramidases (aCDase, nCDase), Cer is hydrolysed to yield free sphingosine (Sph). Sph can be the substrate for either ceramide sintase (CerS), via the **(3)** ceramide salvage pathway or sphingosine kinases (SphK) to form cer and sphingosine-1-phosphate, respectively. CerS, ceramide synthase; DES, dihydroceramide desaturase; GCS, glucoceramide synthase; SL, sphingolipid SMS, sohingomyelin synthase; SPT, serine palmitoyltransferase. Figure taken from rev in. Jenkins *et al.,* 2009.

I.2.2.2. Sphingomyelin cycle

SM hydrolysis, the second Cer-generating pathway, involves the hydrolysis of the phosphocholine head group from SM by a small family of SMases. Cer is then hydrolysed in Sph and fatty acid by the action of ceramidases.

SMases (EC 3.1.4.12) are distinguished by their optimal pH and/or subcellular location. To date several SMases have been characterized: lysosomal SMase (aSMase), zinc ion-dependent secretory SMase (sSMase), neutral magnesium ion-dependent SMase (nSMase), and alkaline SMase (bSMase) (rev. in Marchesini and Hannun, 2004). The breakdown of SM occurs at the outer leaflet of the PM, in association with lipoproteins, and within the endolysosomal system (rev. in Jenkins *et al.,* 2009). Because PM is enriched in SMS2 as well as in aSMase, PM Cer levels are likely to be balanced by the activities of these two enzymes.

Regarding the ceramidases (EC 3.5.1.23), five have been cloned and are categorized as acid, neutral or alkaline ceramidases, depending on the optimum pH of their enzymatic activity being pH 4.5 for acid ceramidase (AC, *ASAH1* gene), pH 7-9 for neutral ceramidase (*ASAH2* gene) and pH 8.5-9.5 for alkaline ceramidases (encoded by *ASAH3/ACER1*, *ACER2* and *ACER3* genes) (rev. in Canals *et al.,* 2011). These distinct ceramidases are found in diverse cellular compartments: AC in the lysosomes (Koch *et al.,* 1996), neutral ceramidase at the PM, intracellular compartments and secreted in the intestinal lumen (El Bawab *et al.,* 2000), and ACER family in the ER-Golgi network (Mao *et al.,* 2001; Xu *et al.,* 2006). Thus, it is thought that ceramidases should have a crucial role in regulating not only the hydrolysis of Cer but also in the formation of Sph and sphingosine-1-phosphate, thereby controlling cellular responses mediated by these bioactive lipids (rev. in Mao and Obeid, 2008). Cer-1-phosphate (Cer1P) is produced by the transfer of phosphate (from ATP) to Cer by Cer kinase, presumably located at the cytosolic leaflet of cellular membranes (Van Overloop *et al.,* 2006).

I.2.2.2.1. Acid sphingomyelinase

Although primarily a lysosomal protein, aSMase can also be secreted through alternative trafficking of a shared protein precursor (pro-aSMase), giving rise to secretory aSMase (sSMase).

The aSMase is first synthesized as a pre-pro-enzyme (75 kDa) representing the full-length, N-glycosylated protein. The pre-pro-aSMase is rapidly processed to pro-aSMase (72 kDa), and within the acidic compartment matures to a 70 kDa form, and lastly is processed to a 52 kDa polypeptide (Hurwitz *et al.,* 1994b). Several evidences suggest that 70 kDa and/or 52 kDa forms represent the lysosomal SMase (Ferlinz *et al.,* 1994; Hurwitz *et al.,* 1994a). aSMase arises from mannose-6-phosphorylation of N-glycans. The precursors that are not mannose-6-phosphorylated get directed to the Golgi secretory pathway and released extracellularly giving rise to sSMase (Schissel *et al.,* 1996, 1998). However, given the lack of investigation into the Zn-requirement of these different forms of aSMase, the precise molecular identity of the mature, Zn-independent aSMase remains unclear.

In addition to its importance in preserving lysosomal function, aSMase has also been reported to be regulated by various cellular stressors to promote Cer formation (rev. in Gulbins and Li, 2006). Consequently, the aSMase/Cer pathway has been considered a major component of the acute cellular stress response (rev. in Zeidan and Hannun, 2010). Its physiological relevance is also illustrated by the severity of the lysosomal disease Niemann-Pick types A (NPA) and B (NPB) which is caused by mutations in the gene encoding aSMase (*SMPD1*). NPA, characterized by neurodegeneration, is the infantile form of the disease and is lethal by the age of 2-3 years. NPB is the later-onset form and distinguished by progressive hepatosplenomegaly, pulmonary insufficiency and heart disease (rev. in Schuchman, 2009). Common between infants with NPA and most patients with NPB is interstitial lung disease caused by SM storage in pulmonary macrophages (rev. in Minai *et al.,* 2000).

I.2.2.3. Lysosomal catabolism

Cer can also be produced by the catabolism of complex SLs, as displayed in Figure 1.6. Through a series of events referred to as SL recycling or the salvage pathway, SM and GSLs are degraded within acidic cellular compartments (LE and lysosomes) by resident enzymes. The catabolism of SLs is ensured by the sequential action of a series of lysosomal hydrolases. The first stage is the step-wise removal of monosaccharides by exoglycosidases from the non-reducing ends of the glycan moieties. Subsequently, the GSLs degradation pathways converge on the next three molecules, LacCer, GlcCer and lastly Cer, which is degraded to Sph (rev. in Winchester, 2005). Unlike Cer, Sph is able to cross the lysosomal membrane to be reutilized or further broken down (Chatelut *et al.,*

1998; Riboni *et al.,* 1998). The Sph can be converted to Cer by Cer synthase (salvage pathway) or phosphorylated by Sph kinase in Sph1P (Smith and Merrill, 1995). Because this is the only way for the formation of Sph1P, the cellular amount of this metabolite is highly dependent on Sph available in the cell. The importance of this pathway is represented by the severity of specific inherited defects, the sphingolipidoses, next described.

I.3. SPHINGOLIPIDOSES

Sphingolipidoses are a class of lysosomal storage diseases (LSDs), characterized by SL deposition due to defects in genes encoding proteins involved in the lysosomal degradation of SLs or their proteinaceous cofactors (Figure 1.6.). Consequently, the SL substrate for the defective protein accumulates in affected cells (rev. in Raas-Rothschild *et al.,* 2004). These diseases were grouped according to the identity of the storage material (rev. in Kolter and Sandhoff, 1998). Their mode of inheritance is autosomal recessive with the exception of Fabry disease which presents an X-linked inheritance pattern (rev. in Desnick *et al.,* 2001).

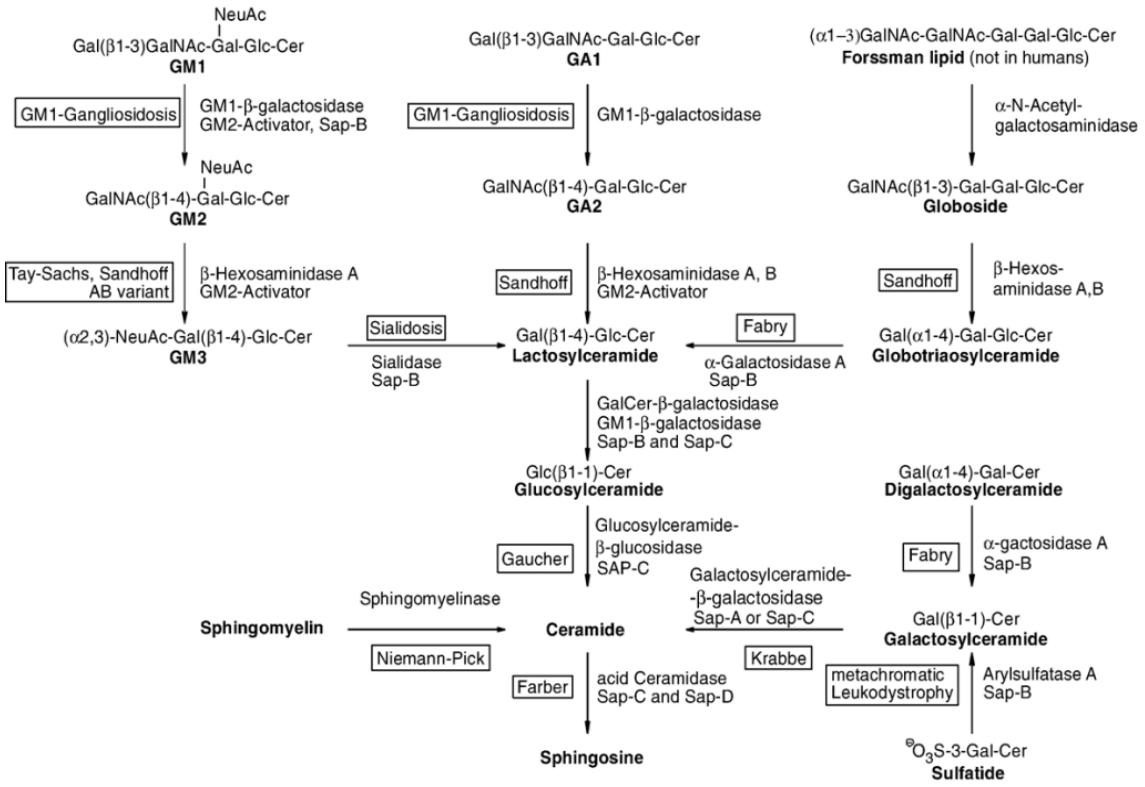

Figure 1.6. Sphingolipid catabolism and sphingolipidoses. Disease-caused enzyme deficiency and activator proteins (SAP) required for the respective degradation step *in vivo* are indicated. Figure taken from rev. in Kolter and Sandhoff, 2006.

Clinical presentation (Table 1.1) varies with the specific subtype of the disease, but common features include neurological symptoms like dementia, major psychiatric illnesses, cerebral palsy, developmental delay, rarefaction of the bones, and several somatic symptoms, such as hepatomegaly and splenomegaly (rev. in Fletcher, 2006; Staretz-Chacham *et al.*, 2009). In addition, detailed medical description can be found in the Online Metabolic and Molecular Bases of Inherited Disease (OMMBID) website.

Sphingolipidoses are rare inborn diseases but highly mortal. Overall frequencies of occurrence of LSDs have been reported for several countries (Meikle *et al.,* 1999; Poorthuis *et al.,* 1999; Applegarth *et al.,* 2000; Dionisi-Vici *et al.,* 2002; Pinto *et al.,* 2004), while other reports focused on LSD groups or on particular LSDs. In certain ethnic groups, as in Ashkenazi Jews, specific LSDs attain a particularly high frequency (Genetics Home Reference). In the general population, the combined incidence of LSDs is estimated to be 1 in 5,000-7,000 live births (Fletcher, 2006; Fuller *et al.,* 2006), being considered the most common cause of pediatric neurodegenerative diseases. Even so, it is believed that the value is underestimated because of the possible undiagnosed or misdiagnosed cases. The prevalence in Portugal is estimated in 1:4,000 live births (Pinto *et al.,* 2004).

I.3.1. Primary alterations

The primary causes sphingolipidoses and major organs involved are described in Table 1.1. The primary substrate that accumulates in the lysosome, i.e. the substrate for the enzyme deficiency, leads to a range of pathology that can involve the liver, spleen, kidney, bone and central nervous system. For all these diseases the associated gene is known and hundred mutations described thus far (rev. in Filocamo and Morrone, 2011; Staretz-Chacham *et al.*, 2009).

Onset and severity of the storage disease is partly determined by the residual activity of the gene product in the lysosomes (rev. in Conzelmann and Sandhoff, 1983,84; Leinekugel *et al.,* 1992). A complete deficiency of a lysosomal enzyme leads to an early onset and a severe course of the disease, whereas only a few percent of residual activity can be sufficient to delay the onset of the disease, cause an attenuated course, and lead to the often misdiagnosed adult forms of the diseases (rev. in Rapola, 1994). Although this is valid for most of the LSDs, patients with identical genotype and, accordingly, formal similar residual activity, can undergo different courses of a disease. Apparently, other genetic and epigenetic factors may contribute to the expression of a disease in an

Table 1.1. Clinical, biochemical and genetic basis of sphingolipidoses.

Disease	Defective protein	Main storage materials	Inheritance and Gene	OMIM No.	# mutation	Major peripheral organ systems affected	CNS pathology
Fabry	α-Galactosidase	Globotriasylceramide Digalactosylceramide	Xq22; *GLA*	301500	517	Kidney, heart	-
Farber	Acid ceramidase	Ceramide	8p22-p21.3; *ASAH1*	228000	23	Skeleton, laryngeal	+
Gangliosidosis GM1	GM1-β-galactosidase	GM1 ganglioside, keratan sulfate	3p21.33; *GLB1*	611458	125	Skeleton, heart	+
Gangliosidosis GM2 Tay-Sachs	β-Hexosaminidase A	GM2 ganglioside	15q24,2; *HEXA*	272800	126	Liver, eyes and brain	+
Gangliosidosis GM2 Sandhoff	β-Hexosaminidase A + B	GM2 ganglioside	5p13; *HEXB*	272800	41	Spleen/liver, brain	+
Gaucher	Glucosylceramidase	Glucosylceramide	1q21; *GBA*	606463	334	Spleen/liver, bone marrow	+[a]
Krabbe	β-Galactosylceramidase	Galactosylceramide	14q31; *GALC*	245200	78	Heart	+
Metachromatic leucodystrophy	Arylsulphatase A	Sulphatide Lactosylsulphatide	22q13; *ARSA*	250100	152	Brain	+
Niemann–Pick (type A, type B)	Sphingomyelinase	Sphingomyelin	11p15.1-p15.4; *SMPD1*	607608	121	Brain[b] Spleen/liver, heart[c]	+[b]

Legend: CNS, Central nervous system; [a]Types 2 and 3; [b]Type A; [c]Type B.

individual patient (rev. in Kolter and Sandhoff, 2006). Such diverse and extensive array of clinical manifestations suggests that many secondary biochemical pathways involving, but not limited to, SLs must also be affected.

I.3.2. Secondary alterations

Although primary enzymatic defects and the resulting clinical phenotype have been well characterized for LSDs, little is known about the sequence of biochemical and cellular events that lead to the pathology.

A characteristic feature of sphingolipidoses is the accumulation of other lipids as secondary storage products (rev. in Walkley and Vanier, 2009). These arise from the lipid nature of the primary storage compounds that co-precipitate other hydrophobic material and produce a kind of traffic jam (rev. in Aridor and Hannan, 2000, 2002). For example, the pathological accumulation of the SL that are not degraded due to mutant enzymes perturbs the cholesterol homeostasis and consequently, changes the composition of the membrane leading to problematic sphingolipid transport (rev. in Pagano, 2003; rev. in Pagano *et al.,* 2000). Thus, several factors, not mutually exclusive, have been implicated in LSDs: lipid trafficking abnormalities, mitochondria dysfunction, autophagy, apoptosis, calcium homeostasis disruption, cellular metabolic stress and inflammation. In the case of sphingolipidoses, cells deficient in lysosomal enzymes or soluble non-enzymatic proteins (e.g. SAP) accumulate excessive levels of undegraded molecules (enzyme deficiency) or catabolic product (efflux permease deficiency) in endo/autolysosomes. The excessive level of these molecules inhibits catabolic enzymes and permeases that are not genetically deficient, resulting in secondary substrate storage (rev. in Walkley and Vanier, 2009; rev. in Platt *et al*., 2012; rev. in Prinetti *et al.,* 2011). The accumulation of primary and secondary substrates triggers a cascade of events that may impact not only the endosomal-autophagic-lysosomal system, but also other organelles, including mitochondria, peroxisomes, nucleus, ER, GC and overall cell function (Figure 1.7).

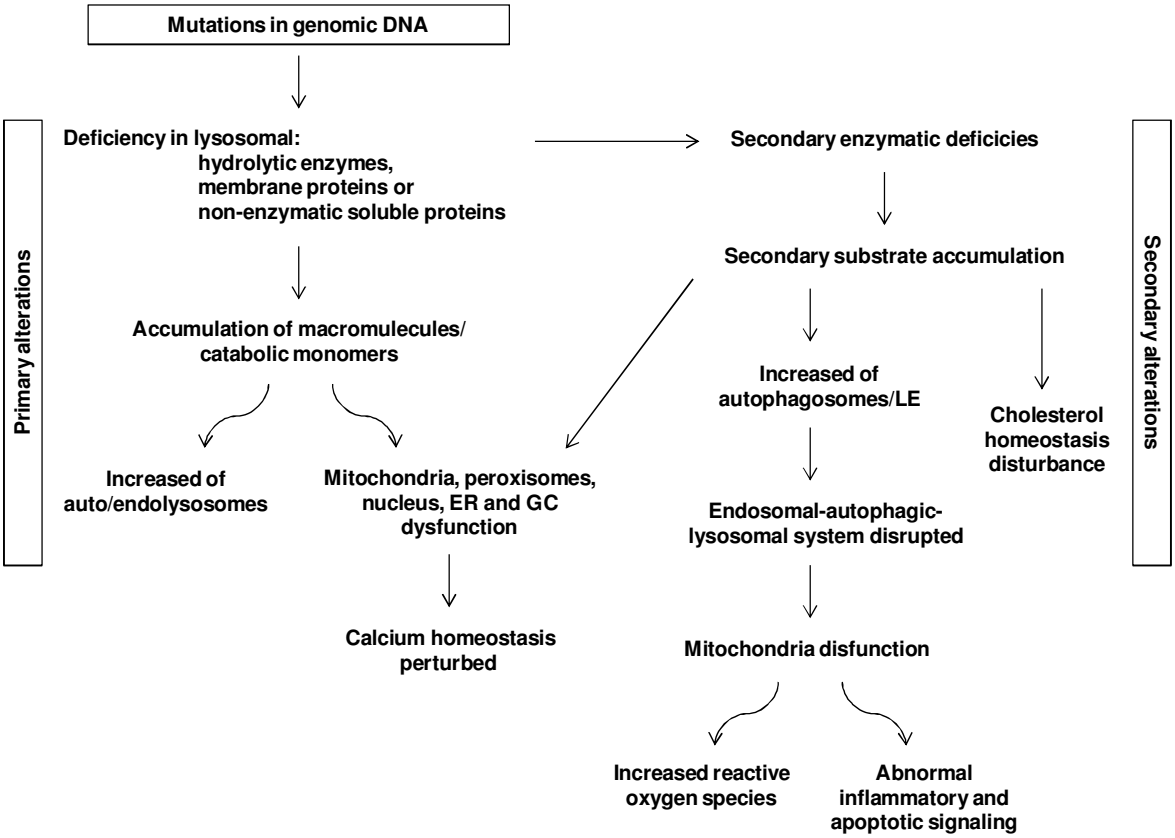

Figure 1.7. Hypothetical cascade of pathological events in LSDs. Mutation in genes coding for lysosomal proteins cause the accumulation of undegraded macromolecules (enzyme deficiency) or monomeric catabolic products (efflux permease deficiency), increasing the number of endo/autolysosomes in cellular cytoplasm. This event can affect enzymes that are not genetically deficient resulting in secondary substrate accumulation. The accumulation of primary and secondary substrates may have impact not only in the endosomal-autophagic-lysosomal system, but also in other organelles, including mitochondria, the ER, GC, peroxisomes and in overall cell function. ER, endoplasmatic reticulum; GC, Golgi complex; LE, late endosomes. Figure adapted from rev in. Platt *et al.,* 2012.

I.3.3. Therapy

Many of the discussed disorders remain untreatable. However, over the past two decades there has been a considerable expansion in the number of therapeutic strategies for LSDs. The theoretical basis for the therapeutic approaches toward sphingolipidoses is the “threshold theory” (rev. in Conzelmann and Sandhoff, 1983,84). According to this theory, the ratio of substrate influx into the lysosomes and the degradation capacity determines the onset and severity of the diseases. Both parameters can be addressed by therapeutic approaches, and the theory predicts that already slight changes in this ratio can improve the condition of the patients (rev. in Kolter and Sandhoff, 2006). The objective of most therapies reported for sphingolipidoses is the restoration of the defective

degradation capacity within the lysosome. In principle, the earlier they are applied, more successful the therapy will be.

Current therapeutic strategies in use or under evaluation are enzyme replacement therapy, bone marrow transplantation, hematopoietic stem cell transplantation, gene therapy, substrate reduction therapy and enzyme stabilization (rev. in Boyd *et al.*, 2013; Desnick and Schuchman, 2002; Gatt and Dagan, 2012; Jeyakumar *et al.,* 2005; Platt and Lachmann, 2009).

I.4. FARBER DISEASE

The deficiency in human AC activity, caused by mutations in *ASAH1* gene, leads to Farber disease (FD, Farber Lipogranulomatosis), a rare LSD with an autosomal recessive mode of inheritance. Less than 100 cases have been reported thus far. This disease is characterized by the accumulation of Cer in several cell types (rev. in Levade *et al.*, 2009).

I.4.1. The *ASAH1* gene

The human *ASAH1* gene (OMIM 613468) spans about 30kb on chromosome 8p21.3-p22 and comprises 14 exon and 13 introns. The full-length cDNA contains an open reading frame of 1185 bp, an 1110-bp 3´untranslated sequence, and an 18-bp poly(A) tail which translation gives rises to a 395 amino acid polypeptide with a calculated molecular mass of this polypeptide being ~44kDa, (Koch *et al.,* 1996; Li *et al.,* 1999). The cDNA encoding murine AC has also been cloned and it was found to have ~80% identity and ~90% homology with human AC (Li *et al.,* 1999). Multiple transcript variants encoding several distinct isoforms have been identified for this gene (Ensembl genome database: ENSG00000104763).

AC expression, assessed to date in only a small number of tissues, was found to be highest in heart and kidney, with moderate expression in placenta, lung and skeletal muscle (rev. in Zeidan *et al.,* 2008).

I.4.2. Biosynthesis and maturation of AC

The maturation of the precursor polypeptide from the ER through the Golgi involves acquisition and processing of 5-6 predicted N-linked glycosylations sites, resulting in the increase of the molecular mass of the polypeptide from the expected 44kDa to 53-55kDa (Ferlinz *et al.,* 2001). The precursor is autoproteolytically processed at the α subunit (13kDa) and β subunit (40kDa), linked by a disulfide bridge at Cys 143, in LE or lysosome (Bernardo *et al.,* 1995; Ferlinz *et al.,* 2001; Shtraizent *et al.,* 2008). Cleavage of the precursor protein is most likely an autoproteolytic event with Cys143, Arg159, and Asp162 as a functional triad. Only the precursor form and the β subunit have N-glycosylation sites and site-directed mutagenesis confirmed the importance of 5 putative N-linked glycosylation sites for proper proteolytic processing and full enzymatic activity (Ferlinz *et al.,* 2001; Schulze *et al.,* 2007). Several experimental evidences obtained by studying the molecular forms of AC present in secretions of cells treated with lysosomotropic agents, I-cell fibroblasts (cells with mutated UDP-N-acetylglucosamine-1-phosphotransferase, the enzyme that catalyzes the formation of M6P in mannose-rich oligosaccharides) and M6P receptor deficient fibroblasts (Ferlinz *et al.,* 2001) suggest that the precursor form of AC is transported to lysosome via M6P pathway.

I.4.3. Protein function

In addition to the hydrolysis of Cer into Sph and free fatty acid, the AC has the ability to synthesize Cer from Sph and fatty acid. However, while the hydrolysis reaction occurs at pH 4.5, the reverse reaction *in vitro* occurs at an optimum pH of 6.0 (Okino *et al.,* 2003), suggesting that both reactions occur in distinct cellular compartments. This possibility would imply a unique mode of regulation of AC enzymatic activity since in some places it could promote the degradation of Cer, and in others its synthesis. However, the possibility that both reactions occur in different extent in the same cell compartment cannot be presently excluded.

The degradation of membrane-bound Cer by AC requires the presence of SAP, such as SAP-D, but also SAP-C and SAP-A. *In vitro* these saponins stimulate Cer hydrolysis in the presence of particular anionic phospholipids such as bis(monoacylglycero)phosphate (BMP) or phosphatidylinositol (Linke *et al.,* 2001). The most effective stimulator, however, is BMP, a lipid that was localized on internal membrane structures of fibroblasts lysosomes (Kobayashi *et al.,* 1998).

Immunoprecipitation studies demonstrated that AC, aSMase and β-galactosidase (Glase) activities in chinese hamster ovary media were co-precipitated using anti-AC antibodies, suggesting that the enzyme can be part of a multienzyme complex that integrates aSMase and Glase (He *et al.* 2003).

I.4.4. Disease's clinical presentation

The first case of FD was described and characterized in 1957 by Sidney Farber (Farber *et al.,* 1957), but only in 1972 the inherited deficiency of AC activity was found to be the cause of this disorder (Sugita *et al.,* 1972).

The clinical presentation of FD is characterized by the appearance of subcutaneous skin nodules, ordinarily near the joints, most often interphalangeal, wrist, elbow and ankle joints, or over points of mechanical pressure. These manifestations are very painful and lead to progressive joint stiffness, limitation of motion by contractures and finally to immobilization and deformation of joints. Also, characteristic signs of FD are the development of a progressive hoarseness due to laryngeal involvement and premature death. Hepatosplenomegaly and nervous system dysfunctions may also occur (rev. in Moser *et al.,* 2001). At nervous system level, the main alteration found in the majority of autopsied patients was the accumulation of storage material in the neuronal cytoplasm (Moser *et al.,* 1969). The lungs are involved in all severely affected patients, with the interalveolar septa and alveoli infiltrated by massive numbers of macrophages. Granulomatous nodules are also found on the parietal pleura. A few patients showed heart involvement with a thickening and nodule formation on the mitral and aortic valves and the chordae tendineae. Lesions are variably found in other sites, including the lymph nodes, intestine, spleen, kidney, tongue, thymus, gallbladder, epithelium, and liver (rev. in Levade *et al.,* 2009).

Based on the clinical severity, age at onset and sites of major tissue involvement, FD has been classified into 7 subtypes. The underlying cause of subtypes 1–5 (classical, intermediate, mild, neonatal, neurologic progression) is the deficiency of AC (rev. in Levade *et al.,* 2009; Park and Schuchman, 2006), while subtype 6 occurs as a result of coincidental combination of FD and Sandhoff disease (Fusch *et al.,* 1989; Levade et al., 1995a) and subtype 7 is due to sphingolipid activator prosaposin deficiency (the precursor protein of four SAPs, A-D), leading to lack of acid ceramidase, β-Galalctocerebrosidase (GalCase) and GlcCase activities (Harzer *et al.,* 1989; Schnabel *et al.,* 1992). This data

demonstrated, for the first time, that AC requires an intact prosaposin gene (Kishimoto *et al.*, 1992) for *in vivo* function.

Subtypes 1 to 3 are the most common forms of the disease. Subtype 1 patients (classical, early infantile onset variant) have a very severe phenotype and most die by 2 years of age. A majority have neurological involvement and lung disease in addition to the characteristic findings mentioned above (rev. in Moser *et al.,* 2001). So far, thirty-five patients presented the classic phenotype (rev. in Levade *et al.,* 2009). Subtypes 2 (intermediate) and 3 (mild) FD patients generally have milder signs and symptoms, living longer than those with subtype 1. They still have granulomatous inflammation leading to subcutaneous nodules, joint pain and contractures, hoarseness, growth deficit, respiratory involvement and intellectual disability, showing only slight or no symptoms of central nervous system disease (rev. in Moser *et al.,* 2001). Twenty-five patients were assigned to the intermediate–mild phenotype (rev. in Levade *et al.,* 2009).

Subtype 4 of FD is an extremely rare, neonatal form of the disease that presents severe neurologic deterioration and large hepatosplenomegaly already in the neonatal period. Only 8 patients with this form have been described (Antonarakis *et al.,* 1984; Kattner *et al.,* 1997; Levade *et al.,* 1995c; Pierpont *et al.,* 1983; Schäfer *et al.*, 1996; Willis *et al.*, 2008) and most severely involved patient presented hydrops fetalis and died on the third day. At this early age, these patients lack the characteristic clinical features of classic FD and, therefore, diagnosis depends on enzymatic and biochemical assays.

Subtype 5 of FD is characterized by progressive neurologic deterioration and, in some cases, tetraplegia, loss of speech, myoclonia, seizures and mental retardation are observed (Eviatar *et al.,* 1986). The survival is up to 5 years of age.

Subtype 6 has been described in only one patient, a 6-month-old girl of consanguineous parents presented with hoarseness, stridor, scattered skin nodules, painful swelling of hand joints and ankles, and cherry-red macular spots. Until the age of 2 years her motor and physical condition deteriorated distinctly, however her mental state remained unchanged. Fibroblasts from this patient presented accumulation of ceramide, profoundly decreased of AC activity as well as total β-hexosaminidase (HexT) activity, corresponding to a biochemically established combination of Farber and Sandhoff disease (Fusch *et al.*, 1989).

Subtype 7 has been reported in four cases, all presenting severe neonatal manifestations and storage of multiple lipids, including Cer (Elleder *et al.,* 2005; Harzer *et al.,* 1989; Hulková *et al.,* 2001; Schnabel *et al.,* 1992).

I.4.5. Molecular basis of the disease

In typical cases of FD, such as subtype 1, the observation of the clinical triad of subcutaneous nodules, joint and laryngeal involvement confirms the disease. The demonstration of typical histopathologic features on biopsy, showing granulomas with macrophages containing lipid cytoplasmic inclusions in subcutaneous nodules or other tissue; or the presence of Farber bodies (comma-shaped curvilinear tubular structures) by electron microscopy are also established diagnostic tests for FD (Faber, 1957; Moser *et al.,* 2001; rev. in Park and Schuchman, 2006; Zarbin *et al.,* 1985). When typical features are missing or laboratorial confirmation is required, diagnosis could be confirmed by the demonstration of reduced AC enzymatic activity and abnormally high Cer levels.

I.4.5.1. Enzymatic deficiency and ceramide storage

The demonstration of reduced AC enzymatic activity can be obtained in cultured skin fibroblasts (Dulaney *et al.,* 1976), white blood cells (Antonarakis *et al.,* 1984), amniocytes (Fensom *et al.,* 1979), plasma (Ben-Yoseph *et al.,* 1989) and postmortem tissues (Sugita *et al.,* 1972), or by loading studies with labeled precursors in cultured cells (Levade *et al.,* 2009).

In AC enzymatic assays, N-oleoyl Sph (Dulaney *et al.,* 1976; Dulaney and Moser, 1977; Fensom *et al.,* 1979; Sugita *et al.,* 1972) was the first and the most commonly used substrate. Then, experiments with the substrate N-lauroylsphingosine (Ben-Yoseph *et al.,* 1989; Momoi *et al.,* 1982) have been performed due to its higher sensitivity and specificity. In 2010, Bedia and colleagues reported a new method for the determination of AC enzymatic activity using the fluorogenic substrate Rbm14-12. This method presents several advantages: the use of a non-radiolabeled and water-soluble analog of Cer as substrate and the stability of the substrate solution of at least 6 months. The assay does not require separation of the product from the substrate, is very sensitive and fast, and requires a few amount of cell lysates, being applicable to a wide variety of biological materials.

The loading tests consists in the addition of exogenous radiolabeled SLs like Cer (Chen *et al.,* 1981; Sutrina and Chen, 1982), sulfatide (Inui *et al.,* 1987; Kudoh and Wenger, 1982), or SM (Levade *et al.,* 1995c), on cultured living cells to study their metabolism. These studies permit the evaluation of the turnover and subcellular compartmentalization of the SLs molecules. Its use in routine procedures is, however,

unfeasible due to its complexity, long execution time and the need of expertise for data interpretation and validation.

Another diagnostic approach is the demonstration of abnormally high Cer levels in cultured cells, biopsy samples or urine, by thin-layer chromatography (Moser *et al.,* 1969; Ozaki *et al.,* 1978), high-performance liquid chromatography (Iwamori *et al.,* 1979; Sugita *et al.,* 1974; Yahara *et al.,* 1980) or gas-liquid chromatography combined with mass spectrometry (Samuelsson and Zetterstrom, 1971), or through the use of the diacylglycerol kinase assay in the presence of $\gamma[^{32}P]ATP$ to measure the radiolabeled Cer1P produced, a 10 times more sensitive technique than the high-performance liquid chromatography (Chatelut *et al.,* 1996).

Mutation analysis should also be considered when the primary defect of the proband is identified and the knowledge of the mutations segregated within a family contribute for a better accuracy of genetic counseling and carrier identification in the families at risk for FD.

I.4.5.2. *ASAH1* gene defects

To date a total of 23 different mutations in the *ASAH1* gene have been found in 20 FD patients of different geographical origins (Table 1.2), mostly missense mutations (17/23).

So far no direct correlation between the genotype, level of residual enzymatic activity, measured *in vitro* in detergent-based assay systems, and the phenotype of FD has been established. However, an inverse correlation between residual Cer turnover, measured in intact cultured cells, and disease severity has been observed (Levade *et al.,* 1995b). Therefore, it is possible that correlation of the disease's clinical severity with the genotype has not yet been properly assessed because only a very limited number of patients have been genotyped thus far.

Table 1.2. Review of the literature: genotype-phenotype correlations.

DNA change	Exon	Protein change	Other allele	Sex	Origin	Onset	Age of Death	Subtype	Consanguinity	AC activity
c.66G>C	1	Q22H	NI	NI	NI	NI	NI	NI	NI	NI
c.67C>G	1	H23D	NI	NI	NI	NI	NI	NI	NI	NI
c.107A>G	2	Y36C	Y36C	M	Tunisian	NA	2y	1	Yes	<5%
c.286_288del	4	V96del	V96del	M	Japonese	8m	6,3y	2	Yes	37%
c.290T>A **c.703G>C**	4 9	V97E G235R	G235R V97E	F	Japonese	10m	>10y	2//3	No	35% 2%
c.290T>G	4	V97G	V97G	M F	Saudi Arabia	3m 9m	NI	1	Yes	NI
c.383-457del **c.412G>T** **c.991G>A**	6 12	G128_K152del; E138X D331N	D331N G128_K152del; E138X	F	NI	NI	>1.5 y	3	No	NI
c.413A>T **c.383-457del**	6	E138V G128_K152del;	E138V G128_K152del;	F	Sicilian	20m	30y	3	NI	5%
IVS6+4A>G	NI	NI	NI	F	Indian	1y	1,3y	1//5	Yes	NI
c.502G>T	7	G168W	G168W	M	Croation	2w-4m	38,5m	1	No	NI
c.544C>G	8	L182V	L182V	F M	Indian	1m NI	6m 2y	1	Yes	NI
c.533 T> C **c.1144 A> C**	8 13	W185R K382Q	NI NI	F	Emirati (Balochi)	8m	-	2	No	NI
c.665C>A	9	T222K	T222K	F	Belgium	NI	22m	1	Yes	5%
c.760A>G	10	R254G	NI	NI	NI	NI	3y	1	NI	<10%
c.833C>T	11	P278L	P278L	M	NI	10m	>1.3	2/3	NI	NI
c.958A>G	12	N320D	N320D	F	Turkish	2,5y	6y	6	Yes	10%
IVS13+1G>T	13	N348_K366del	NF	M	NI	NI	3y	5	NI	<15%
c.1085C>G	13	P362R	P362R	NI	NI	NI	1,5y	1	Yes	<5%
c.1085C>G	13	P362R	NI	NI	NI	NI	8y	2/3	NI	NI
c.1187insT	14	X396L	NI	NI	NI	NI	NI	NI	NI	NI

Legend: del, Deletion; F, Female; M, Male; m, Months; NI, Not indicated; y,Years.

I.4.6. Pathophysiology

As mentioned above, FD is a very rare autosomal recessive disorder, and less than 100 cases have been reported since the first description in 1957. Due to the extremely small patient population, the natural history and underlying pathophysiology of this disease is poorly described.

The main pathologic feature of disease appears to be the accumulation of macrophages causing granulomatous infiltrations in the subcutaneous tissues, joints, liver, spleen, lymphoid tissue, thymus and lungs (Antonarakis *et al.,* 1984). The etiology of FD is the lack of acid ceramidase, and subsequently there is an increased storage of Cer in several organs and tissues (Moser *et al.,* 2001). The principal symptoms of disease, mainly in the patients without involvement of the central nervous system, are caused by dysregulation of leukocyte functions (Burek *et al.,* 2001), implicating the dysfunction of the immune system that results in the inflammatory component of the disorder with painful swelling of several joints, granuloma formation, contractures and inflammatory airway involvement (rev. in Ehlert *et al.,* 2007). Of note, recently *ASAH1* gene mutations have been described in patients with spinal muscular atrophy associated with progressive myoclonic epilepsy (Zhou *et al.,* 2012). Nevertheless, the molecular mechanisms underlying the Cer accumulation to chronic granulomatous inflammation is not yet fully understood.

Cer has been shown to be implicated in many cellular events including response to stress (rev. in Andrieu-Abadie *et al.,* 2001; rev. in Hannun and Obeid, 2002), regulation of cell growth and differentiation (rev. in Bieberich, 2011), cell migration and adhesion (Hla, 2004) and cell senescence (Webb *et al.,* 2010). Roles for Cer and its downstream metabolites have also been suggested in a number of pathological states including cancer (Ponnusamy *et al.,* 2010; rev. in Zeidan *et al.,* 2008), neurodegeneration (rev. in Jana *et al.,* 2009), obesity, diabetes (Amati *et al.,* 2011; rev. in Bikman and Summers, 2011), immune inflammatory responses (rev. in Pandey *et al.,* 2008) and apoptosis (rev. in Mullen and Obeid, 2012). How these bioactive functions of Cer could be directly relevant to the Farber pathogenesis remains ambiguous, since these signaling pathways appear to involve specific non-lysosomal pools of Cer (Birbes *et al.,* 2001; Zhang *et al.,* 1997). As a matter of fact, studies performed by Tohyama and colleagues (1999), indicated that despite the levels of Cer in cultured skin fibroblasts of four Farber patients were 2.9 fold higher, these cells do not exhibit different apoptotic features comparing to control cells. In another study, fibroblast or lymphoid cell types of FD patients stimulated with TNF-α, anti-CD95 or CD40 antibodies, anthracycline, or ionizing radiation, as well as cell permeant

ceramides, underwent apoptosis just as control cells (Ségui *et al.,* 2000). These results agree with the notion that FD is characterized clinically by symptoms that do not evoke any abnormality of apoptotic processes (Moser, 1995). Furthermore, overexpression of AC had no effect in cell viability or in stress-induced apoptotic response (Ségui *et al.,* 2000). Taken together, these findings strongly argue against endosomal/lysosomal ceramide playing a role in stress-induced apoptosis and further strengthen the notion that the Cer produced in cells stimulated by apoptotic agents does not result from an endosomal/lysosomal SMase activity (Bezombes *et al.,* 2001; Ségui *et al.,* 2000). Indeed, there are different intracellular pools of Cer and its production appears to be highly compartmentalized, namely at the cell surface, in caveolae, from which it can be delivered to specific sites of biomodulating action within the cell (Liu and Anderson, 1995). Therefore, the Cer accumulated in Farber cells might be without biomodulating effect on its cellular targets due to its localization in the lysosomes. If an extra-lysosomal accumulation of a signaling pool of Cer occurs in FD is presently unclear.

I.4.7. Therapy

Currently, the therapeutic management of FD is mainly palliative. It has been focused on pain therapy, physical therapy, surgical correction of severe contractures and anti-inflammatory medication. The affected patients usually die in the second decade of their lives as a consequence of granuloma formation in the respiratory tract, their immobility with recurrent episodes of pneumonia and resulting respiratory insufficiency (Moser *et al.,* 2001). However, some advances have been made, especially in the last decade.

The first reports on allogeneic hematopoietic stem cell transplantation in FD patients were published in 1989 and 2000 by Souillet and Yeager. The two patients with the classic FD phenotype subjected to treatment showed granulomas regression, a diminished joint discomfort, improved joint mobility, and hoarseness had diminished. However they died because of a deterioration of their neurological status, as in other lysosomal storage disorders with severe CNS disease. Starting in 2001, 3 children and 1 adult with FD, presenting minimal CNS involvement, have been transplanted so far. High levels of donor chimerism were observed, and all 4 patients showed significant decrease in the number of granulomas as well as a general improvement in joint mobility with subsequent reduction in pain (rev. in Ehlert *et al.,* 2007; Vormoor *et al.,* 2004). Thus, hematopoietic stem cell transplantation has showed some promising results on patients with minimal central nervous system involvement.

In 1999, the feasibility of gene therapy at the cellular level for FD, were tested. The authors, created a novel recombinant retrovirus for the transfer of the human AC cDNA and observed that the retrovirus mediated expression of AC in skin fibroblasts was accompanied not only by the increase in enzyme activity, as detected *in vitro* in transduced fibroblast extracts, but also by normal lysosomal turnover of Cer. This increased catabolism of Cer led to the complete correction of the metabolic defect as demonstrated by the reduction of accumulated intracellular Cer to normal levels (Medin *et al.,* 1999). In more recent studies, *in vitro* assays with novel recombinant retrovirus and lentivirus led to the same successful results (Ramsubir *et al.,* 2008). Then, murine models were developed to apply the viral vectors *in vivo*. The first model used was xenografts of vector-transduced human hematopoietic cells. The second model incorporated direct therapeutic lentivector injection into neonatal mice. In both cases, vector-transduced cells persisted in mice, and long-term transgene expression was evident over time (Ramsubir *et al.,* 2008). The last advances in gene therapy approach, was the employing of lentivirus transduction and transplantation of primary hematopoietic cells in a non-human primate model, the rhesus macaques (Walia *et al.,* 2011). The animals were followed for at least 1 year post-transplantation to track the sustainability of the transgene in peripheral blood and bone marrow cells. Increases of AC enzymatic activity to supra-normal levels in peripheral blood cells, bone marrow cells, spleen, and liver were observed throughout the study period. Additionally, Cer levels were reduced due to increased AC activity even in these metabolically normal animals. These results show that transplantation of lentivirus transduced mobilized peripheral blood cells is safe and may be efficacious for FD and, by extension, other LSDs.

Chapter II

Aims of the study

Farber disease is a clinically heterogeneous autosomal recessive disease caused by mutations in the *ASAH1* gene. This gene codes for acid ceramidase, a lysosomal heterodimeric enzyme that acts in the last step of the sphingolipid degradation pathway by hydrolysing ceramide into sphingosine and fatty acid.

In the last two decades, the identification and characterization of FD cell models have provided important insights into the molecular pathogenesis by extending the knowledge of the primary defects underlying the disease. Additionally, they have also been useful to define basic properties of acid ceramidase through the study of its biosynthesis, processing and maturation in the route to lysosomes, and also the bioactivity of lysosomal ceramide storage. Nonetheless, little is known about secondary or tertiary alterations responsible for disease's signs or symptoms and, ultimately, associated with the disease onset and progression. Indeed, the correlation between the clinical phenotype and either the residual acid ceramidase enzymatic activity or the level of ceramide storage is still a controversial issue. Furthermore, very few causative mutations have been reported in the *ASAH1* gene and for most of them the molecular mechanism is unclear, hampering the establishment of genotype-phenotype correlations. In addition, the disruption of *ASAH1* gene in mice was found to cause an early embryonic lethal phenotype, underscoring the importance of acid ceramidase *in vivo* for proper cell and organismal function but precluding its use in the study of the disease's pathobiology. Therefore, the aim of this study was to characterize a unique cellular model underlying the most severe form of FD identified to date by using genetic, molecular and biochemical approaches.

Chapter III

Materials and Methods

This chapter describes the materials and methods used in the studies presented in this thesis.

III.1. PATIENTS

The clinical phenotype and genetic alterations reported for the patients included in the present study are described in Table 3.1.

Table 3.1. Clinical, pathological and mutational spectrum of patients.

Patient	Origin	Sex	Genotype	Mutated protein	Fenotype	Onset	Age of death
FD1	German	Female	present study	present study	Subtype 4 (neonatal)	0,5y	3d
FD2 (GM20015)	Tunisian	Male	[c.107A>G]	[p.Y36C]	Subtype 1 (classic)	0,5y	2y
FD3 (GM20018)	Belgium	Female	[c.665C>A]	[p.T222K]	Subtype 1 (classic)	1y	2y
FD4 (GM02315)	Italian	Female	[c.413A>T]+[c.383_457del]	[p.E138V]+[p.G128_K152del]	Subtype 3 (mild)	2y	30y
NPB1	Portuguese	NI	[c.1426C>T]+[c.2257T>C]	[p.R476W]+[p.C227R]	Type B	NI	NI
NPB2	Portuguese	NI	[c.1426C>T]	[p.R476W]	Type B	NI	NI
NPB3	Portuguese	NI	[c.1829_31delGCC]	[p.R610del]	Type B	NI	NI
NPB4	Portuguese	NI	NI	NI	Type B	NI	NI
NPC	Portuguese	NI	[c.530G>A]+[c.3591+1G>A]	[p.C177Y]	Type C1	NI	NI
MLII	Portuguese	NI	[c.3503_4delTC]	[p.L1168fsX1172]	Type II	NI	NI

Legend: FD, Farber disease; d, days; NPB, Niemann-Pick disease type B; NPC, Niemann-Pick disease type C NI, Not Indicated; MLII, Mucolipidosis type II; y, years.

FD1 patient is the first, and to our knowledge the only, reported FD case with non-immune hydrops fetalis (Kattner *et al.,* 1997; Schäfer *et al.,* 1996). The female patient presented a severe, generalized edema and extreme enlargement of her liver and spleen. Massive histiocytic infiltration was observed in liver, spleen, bone marrow, lymph nodes, thymus, parotid gland and adrenal medulla. The child died 3 days after birth from disseminated intravascular coagulation. The diagnosis of FD, subtype 4, was based on

the deficient enzymatic activity of AC (Kattner *et al.,* 1997) and accumulation of Cer in cultured fibroblasts (Chatelut *et al.,* 1996) and visceral organs (Schäfer *et al.,* 1996).

III.2. BIOLOGICAL SAMPLES AND CELL CULTURE

In this work, cultured skin fibroblasts from patients with FD, Niemann-Pick disease type B (NPB), Niemann-Pick disease type C (NPC), Mucolipidosises type II (MLII) and controls, were used.

Cell line FD1, corresponding to transformed fibroblasts by the human simian virus 40 (SV40) large T antigen, and FD1-AC cells, resulting from FD1 cells transfected with a recombinant retroviral vector cloned with human *ASAH1*/cDNA (AC-transduced cells), were previously described (Chatelut *et al.,* 1996; Medin *et al.,* 1999) and kindly provided by Prof. Thierry Levade (Laboratoire de Biochimie Métabolique, Institut Fédératif de Biologie, CHU Purpan, Toulouse, France). Cell lines FD2, FD3 and FD4, were obtained from Coriell Institute for Medical Research Cell Line Repository (New Jersey, USA).

NPB, NPC, MLII, and control fibroblasts were kindly provided by Dr. Lúcia Lacerda (Biochemical Genetic Unit, Centre Medical Genetics, INSARJ, Porto, Portugal). Skin fibroblasts from control individuals were maintained anonymous throughout the study.

All cell lines were grown to confluency in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% of fetal calf serum and 10% of Penicillin/Streptomycin solution, at 37ºC in an atmosphere containing 5% CO_2. After trypsinization the cells were washed with cold phosphate buffered saline (PBS) and the pellets were frozen at -20ºC or immediately processed. All the cell culture reagents were purchase from Gibco®.

III.3. MUTATIONAL SCREENING IN GENOMIC DNA

III.3.1. *ASAH1* gene

Genomic DNA was extracted from skin fibroblasts with ‘‘EZ1 DNA Tissue kit’’ on BioRobot EZ1 (Qiagen), according to manufacturer´s instructions. Genomic fragments (between 223 and 613 bp), mostly encompassing each exon and the corresponding

flanking sequence, were amplified using the forward and reverse primers listed in Table 3.2. Polymerase chain reaction (PCR) amplifications were performed in a total reaction volume of 25µl using about 100ng of genomic DNA, 1x PCR ImmoMixTM Red (Bioline) and 0.25µM of each primer, for 35 cycles at specific annealing temperatures. After agarose gel electrophoresis, PCR products were purified with ExoSap-IT (Exonuclease I, shrimp alkaline phosphatase; GE Healthcare) according to the manufacturer's instructions and sequenced in forward and reverse directions with BigDye Direct Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 310 automatic sequencer (Applied Biosystems). Raw data was analyzed with the sequence analysis software FINCH TV (Geopiza) version 1.4.0. Comparative sequence analysis was performed using the Ensembl genome server. Each candidate mutation was confirmed in two independent PCR and sequencing reactions.

III.3.2. *SMPD1* gene

The mutational screening in the *SMPD1* gene was performed similarly as described for *ASAH1* gene. Genomic fragments (between 227 and 879 bp), mostly encompassing each exon and the corresponding flanking sequence were amplified using the forward and reverse primers listed in Table 3.3.

III.4. LONG-RANGE PCR AND SEQUENCING

Long-range PCR was performed using the Expand Long Template PCR System (Roche, Applied Science) according to manufacturer's instructions. The specific set of primers used to amplify the target genomic region of the *ASAH1* gene was 5' CAGACTTGATGTTGTCTTCACA-3' (intron 2) and 5'-CTGAAATTTGGGCTTGCTATG-3' (intron 6). The amplified fragment of 11329 bp corresponds to the nucleotides 8371-19699 of the *ASAH1* transcript sequence ENST00000262097. After preheating of 94°C for 2 min, samples were subjected to 30 cycles of denaturation at 94°C for 10 s, annealing at 54°C for 30s, and primer extension at 68°C for 8 min. In the last 20 cycles, successive increments of 20s were applied to the extension time. The final extension was completed by 8 min at 68ºC. After electrophoresis on a 2% agarose gel, the long-range PCR fragment was purified with ExoSap-IT and sequenced on an ABI Prism 310 automatic sequencer (Applied Biosystems). The mutation was confirmed by repeat PCR and sequencing.

III.5. cDNA MUTATION ANALYSIS

III.5.1. *ASAH1* gene

Total RNA was isolated from skin fibroblasts using the High Pure RNA Isolation Kit (Roche Applied Science). First-strand cDNA was synthesized using the Ready-To-Go™ You-Prime First-Strand Beads (GE Healthcare), according to the manufacturer's instructions and PCR amplified in three overlapping fragments, using three sets of specific forward and reverse primers listed in Table 3.2, at specific annealing temperatures. PCR fragments were subjected to sequence analysis using the reference sequence from the Ensembl genome database.

III.5.2. *SMPD1* gene

cDNA mutation analysis of *SMPD1* gene was performed similarly as described for *ASAH1* gene. First-strand cDNA was PCR amplified in five overlapping fragments, using five sets of specific forward and reverse primers listed in Table 3.3.

III.6. SEMI-QUANTITATIVE STUDIES

ASAH1/cDNA fragments were amplified in 25-cycles PCR reaction using specific primers corresponding to the amplification of the fragment 3 (Table 3.2) and analyzed by agarose gel electrophoresis using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as endogenous reference gene (F5'-^{346}GCCAAAAGGGTCATCATCTC^{365}-3' and R5'-^{504}TGGTATCGTGGAAGGACTCA^{523}-3' set of primers). Comparative analysis was performed using ImajeJ software (version 1.6.0).

Table 3.2. *ASAH1* gene: forward and reverse primers.

*ASAH1*_gDNA	Primer Foward and Reverse (5´- >3´)	bp	A.T (ºC)
Exon 1	$^{(-74)}$TTCTTTGCCTCTGCTGGAGTCCGGGGAGT$^{(-46)}$ ^{151}GTGCTGGGCAGCTTCTTTAC^{170}	244	62
Exon 2	^{8345}CAGACTTGATGTTGTCTTCACA^{8366} ^{8684}TCCGATTCAAATGCCCGTCG^{8703}	359	58
Exon 3	^{12521}TGGGTAACCAAATTCTACACTGC^{12543} ^{12738}GCTATGATGCTTAAATCTTTTATTTCT^{12764}	481	58
Exon 4	^{13986}GATGGTGCTACTGCACTCCA^{14005} ^{14318}GCAACCTCTTCGCAGGGACT^{14337}	281	64
Exon 5	^{16625}GTCCAAGAACTAGATTTCAAAGCA^{16648} ^{17014}AGCCCAGCACTCCACGATGC^{17033}	613	62
Exon 6	^{19392}GGCCAAAGCACACACTCACAC^{19412} ^{19653}CTGAAATTTGGGCTTGCTATG^{19673}	282	55
Exon 7	^{20741}GTCCAGCTTTGATTTAGAATC^{20761} ^{20963}TGGTGAAACCCCGTCTCTAC^{20982}	242	58
Exon 8	^{21495}TTGATCGTGGTGACTCGGTAA^{21515} ^{21795}TGTCCTGTTACCGTTATGTAA^{21815}	321	58
Exon 9-10	^{22240}TTACATAACGGTAACAGGAC^{22259} ^{22735}CCAGCTTAATCCTTTGAGGA^{22754}	515	58
Exon 11-12	^{24226}CAGCACATGTGTCCCAGAAC^{24245} ^{24748}GTTCTGGCATTTTTCTTTGG^{24767}	542	58
Exon 13	^{25029}TGGAGGGGAGGTTGATTAAA^{25048} ^{25232}AGGGAAAGAATTTGGCCCTA^{25251}	223	58
Exon 14	^{26170}ACAGTGATCCTGTT^{26183} ^{26557}ACCGAACACTGCAGCTGTCT^{26575}	408	58
***ASAH1*_cDNA**	**Primer Foward and Reverse (5´- >3´)**	**bp**	**A.T (ºC)**
Fragment 1	$^{(-13)}$GGCTGCTAGAGCGATGCCG^{6} ^{1172}CTTGTATAGGTTGGTGAGCA^{1191}	1204	58
Fragment 2	$^{(-230)}$GGTAGCCTGGAAGGCTCTCT$^{(-211)}$ ^{614}CTGGCTATGTGGGCATGTTA^{633}	862	58
Fragment 3	^{488}TTGGAGTATTTCTTGGGTGG^{507} ^{1290}CGGCAGGTTCTCTTTGAGTC^{1309}	822	58

Legend: A.T, annealing temperature; bp,Base pairs; cDNA, complementary DNA; gDNA, genomic DNA. Superscript numbers, position in the DNA considering the A nucleotide of the first ATG as 1.

Table 3.3. *SMPD1* gene: forward and reverse primers.

***SMPD1*_gDNA**	**Primer Foward and Reverse (5´- >3´)**	**bp**	**A.T (ºC)**
Exon 1A	$^{(-199)}$CCACCGAGAGATCAGCTGTC$^{(-180)}$ ^{380}GCTGATGCTGGTGCGCTG^{397}	595	60
Exon 1B	^{171}TCCGGCAGAGGCTCACCCTCTTTC^{194} ^{380}GCTGATGCTGGTGCGCTG^{397}	227	60
Exon 2	^{740}CTCTGCTCTGCCTCTGATT^{758} ^{1565}GAATGAAAGTGAAGGGAG^{1582}	879	55
Exon 3	^{2541}ACCCTCCACCCAAATGCC^{2558} ^{2870}GTTTTATTTTCCTGGCATTCC^{2890}	350	55
Exon 4	^{2870}GTTTTATTTTCCTGGCATTCC^{2890} ^{3133}AGTGTCTGAAGGCTGAAA^{3150}	346	55
Exon 5	^{3243}CAGATGTCTTCCTACCCC^{3260} ^{3479}AGTTGGTGGGATAGGGGAA^{3497}	255	55
Exon 6	^{3518}GCAAAGCATGGGCAGGAT^{3534} ^{4067}AATGCTGCTGTGGTTCAAC^{4085}	568	60
***SMPD1*_cDNA**	**Primer Foward and Reverse (5´- >3´)**	**bp**	**A.T (ºC)**
Fragment 1	$^{(-162)}$GAAGGGGCGGAGCTGCTT$^{(-145)}$ ^{281}GCAAAGGTCTATTCACCGCC^{300}	461	58
Fragment 2	^{182}CTCACCCTCTTTCTCCCC^{199} ^{751}AAGTGTGACCTGCCCCTG^{768}	587	58
Fragment 3	^{651}GGGCACGGACCCTGAC^{666} ^{1243}GCTGAGGATCGAGGAGAC^{1260}	610	58
Fragment 4	^{1170}GAACTTCTGGCTCTTGATCAA^{1190} ^{1988}GGTGAAAGAACCAGTCCCT^{2006}	837	58
Fragment 5	^{1654}CCTACCGCCTGGCACAAC^{1671} ^{1988}GGTGAAAGAACCAGTCCCT^{2006}	353	58

Legend: A.T, Annealing temperature; bp,Base pairs; cDNA, Complementary DNA; gDNA, Genomic DNA. Superscript numbers, position in the DNA considering the A nucleotide of the first ATG as 1.

III.7. BIOINFORMATIC ANALYSIS

Alignments of the nucleotide sequences of the mutant and normal *ASAH1* alleles were achieved using the Ensembl genome server. The web tool Human Splicing Finder (Desmet *et al.,* 2009), version 2.4.1, was used to predict the strength of 5' splice junctions in the *ASAH1* gene, and potential branch points, splicing enhancers and splicing silencers motifs. Splice prediction by using consensus sequences was also performed with softwares SpliceView and MaxEnt (Yeo and Burge, 2004). The nucleotide sequences of the *ASAH1* gene were screened for the presence of repetitive elements using the default settings of the RepeatMasker software, version open-4.0.1. The sequence was also analyzed with the non-B DNA motif search tool (nBMST), version 2.0. (Cer *et al.,* 2013).

III.8. ANTIBODIES

Anti-AC mouse monoclonal antibody raised against the peptide segment 88-182 of the human protein (MCA) was from BD Transduction Laboratories (BD Bioscience). Anti-AC goat polyclonal antibody produced against an internal segment of the human protein (PCA) was from Santa Cruz Biotechnology. Rabbit polyclonal anti-AC antibody (SHA) was kindly provided by Prof. Konrad Sandhoff (Life and Medical Sciences Institute, University of Bonn, Germany). Anti-mouse and anti-rabbit secondary antibodies were from Cell Signalling. Anti-goat secondary antibody was from Santa Cruz Biotechnology.

III.9. PROTEIN ANALYSIS BY WESTERN BLOT

Cell pellets from cultured fibroblasts were suspended in 1x lysis buffer (Cell Signalling), 1mM phenylmethanesulfonylfluoride and protein inhibitor cocktail (Roche), lysed by sonication on ice, followed by a 10 min centrifugation at 13,200g at 4ºC. Under reducing conditions, the samples were prepared with Laemmli sample buffer and 0.1M dithiothreitol. After heat denaturation, approximately 50 µg of protein was electrophoresed on 10% acrylamide gel and transferred to nitrocellulose membrane (Amersham Biosciences). The membrane was incubated in blocking buffer (1 x Tris-buffered saline, 0.1% Tween-20 and 5% dried non-fat milk) for 2h, and incubated overnight at 4°C with primary antibody (diluted in blocking buffer). Immunoreactivity was visualized with horseradish-peroxidase-conjugated secondary antibody followed by chemiluminescence detection (Luminata Western HRP Substrates, Millipore).

III.10. *IN VITRO* ENZYMATIC ACTIVITIES

The AC activity was determined against the artificial fluorogenic substrate, Rbm14-12, kindly provided by Prof. Thierry Levade (Laboratoire de Biochimie Métabolique, Institut Fédératif de Biologie, CHU Purpan, Toulouse, France), as previously reported (Bedia *et al.,* 2010). The aSMase activity was determined against the artificial fluorogenic substrate, the 6-hexadecanoylamino-4-methylumbelliferylphosphorylcholine (HMU-PC; Moscerdam, Netherlands), as previously described (van Diggelen *et al.,* 2005), or against the natural radioactive substrate, choline-methyl-^{14}C sphingomyelin (specific activity 54 mCi/mmol; PerkinElmer, California, USA) as reported (Graber *et al.,* 1994; Jaffrézou *et al.,* 1996; Wiegmann *et al.,* 1994). The activity of β-galactocerebrosidase (GalCase), β-glucocerebrosidase (GlcCase), total β-hexosaminidase (HexT), and β-galactosidase (Glase) was assayed with fluorogenic substrates labeled with 4-methylumbelliferyl: 6-hexadecanoylamino-4-methylumbelliferyl- β-galactoside (Moscerdam), 4-methylumbelliferyl- β-D-glucopyranoside (Glycosynth); 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Sigma) and 4-methylumbelliferyl-β-D-galactopyranoside (Sigma), respectively, as previously described (Ho and O´Brien, 1971; Ribeiro *et al.,* 1991; Wiederschain *et al.,* 1992; Sá Miranda *et al.,* 1990).

III.11. SPHINGOLIPID QUANTITATION ASSAYS

For Cer quantification, lipids were extracted and Cer mass measured as previously reported using *Escherichia coli* membranes as a source of diacylglycerol kinase (Preiss *et al.,* 1987; Van Veldhoven *et al.,* 1992). Radioactive ceramide-1-phosphate was isolated by thin layer chromatography (TLC) (Whatman LK6D plates) using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, v/v/v/v/v). TLC plates were exposed to X-ray films. Then, lipid spots were scraped, and phosphorylated Cer and diacylglycerol were counted by liquid scintillation. SM was quantified by analysis of lipid phosphorus after alkaline methanolysis as previously described (Ames, 1966).

III.12. STATISTICAL ANALYSIS

Data are expressed as mean values ± SD of at least three independent experiments. Values were compared by Student's t-test. The 0.05 probability level was chosen as the point of statistical significance.

Chapter IV

Results

The intent of this chapter of the thesis is to provide an overall and integrated perspective of the achieved results obtained in the course of the PhD project.

IV.1. ELUCIDATION OF THE GENOTYPE OF THE NEONATAL FORM OF FARBER DISEASE

A German patient, representing the first, and to our knowledge the only, reported FD case with non-immune hydrops fetalis (Kattner *et al.,* 1997; Schäfer *et al.,* 1996) was investigated for elucidation of the mutations underlying this extremely severe and rare Farber's phenotype. The diagnosis of FD, subtype 4, has been previously achieved based on the deficient enzymatic activity of AC (Kattner *et al.,* 1997), and accumulation of Cer in cultured fibroblasts (Chatelut *et al.,* 1996) and visceral organs (Schäfer *et al.,* 1996).

IV.1.1. Mutational analysis in gDNA and cDNA of *ASAH1* gene

IV.1.1.1. Identification of the novel mutation g.24491A>G in intron 11

The genomic DNA from FD1 patient´s fibroblasts was screened for mutations by direct sequencing of PCR products corresponding to each of the 14 exons of the *ASAH1* gene and the corresponding intronic flanking sequences. The transition g.24491A>G (c.917+4A>G) in intron 11 was found in heterozygosity (Figure 4.1.A). This novel mutation was not detected in 100 normal control chromosomes (data not shown), discarding the possibility of representing a common panethnically polymorphism. No other predicted deleterious alteration was found in exons and flanking intron sequences of the patient's sample. In accordance with patient's genotype, the mother was heterozygous for the same gene defect (Figure 4.1.B), and the father's sequence normal in this region (Figure 4.1.C).

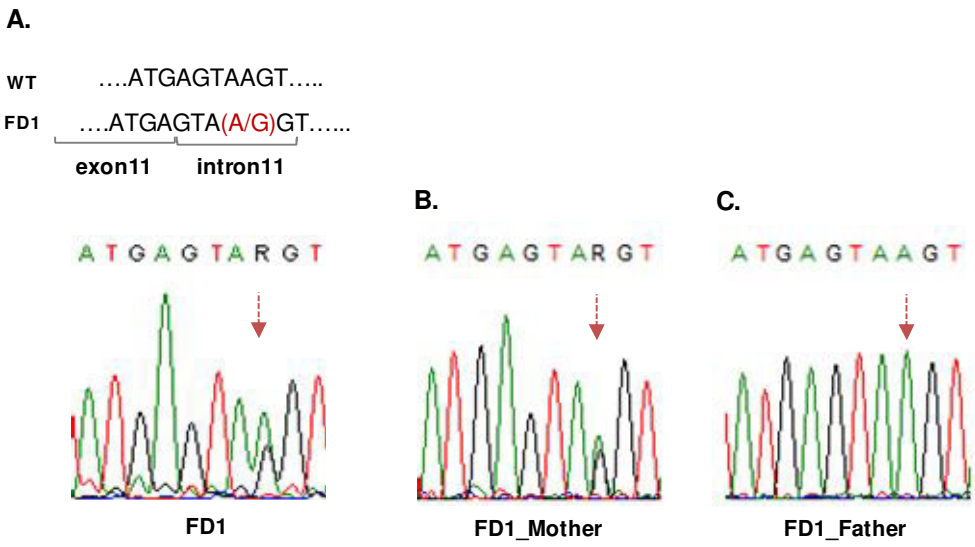

Figure 4.1. Identification of the mutation g.24491A>G. Cycle sequencing of the PCR products obtained from genomic DNA of FD1 patient (**A**) and parents (**B** and **C**) cells was performed as described in the Materials and Methods. The region of the electropherogram encompassing the exon 11/intron 11 junction is indicated by an arrow. FD1, Farber disease patient 1; WT, Normal wild-type sequence.

Since the study of genomic DNA allowed the identification of only one mutant allele, cDNA / *ASAH1* sequencing studies were then carried out.

IV.1.1.2. *ASAH1* / cDNA sequencing revealed the skipping of exons 3 to 5

The cDNA obtained from total RNA isolated from FD patient's fibroblasts was amplified in 3 overlapping fragments and analyzed by agarose gel electrophoresis (Figure 4.2). For the PCR product corresponding to the full length cDNA fragment, a faster migrating band than the wild-type fragment was observed after agarose gel electrophoresis (Figure 4.2.B, fragment 1). Similar finding was obtained for the PCR fragment encompassing exons 1-8 (Figure 4.2.B, fragment 2). For the cDNA-PCR fragment comprising exons 8 to 14 (Figure 4.2.B, fragment 3) the electrophoretic migration pattern found in the patient was comparable to the control. For the most prominent cDNA-PCR bands, DNA was purified and subjected to sequence analysis.

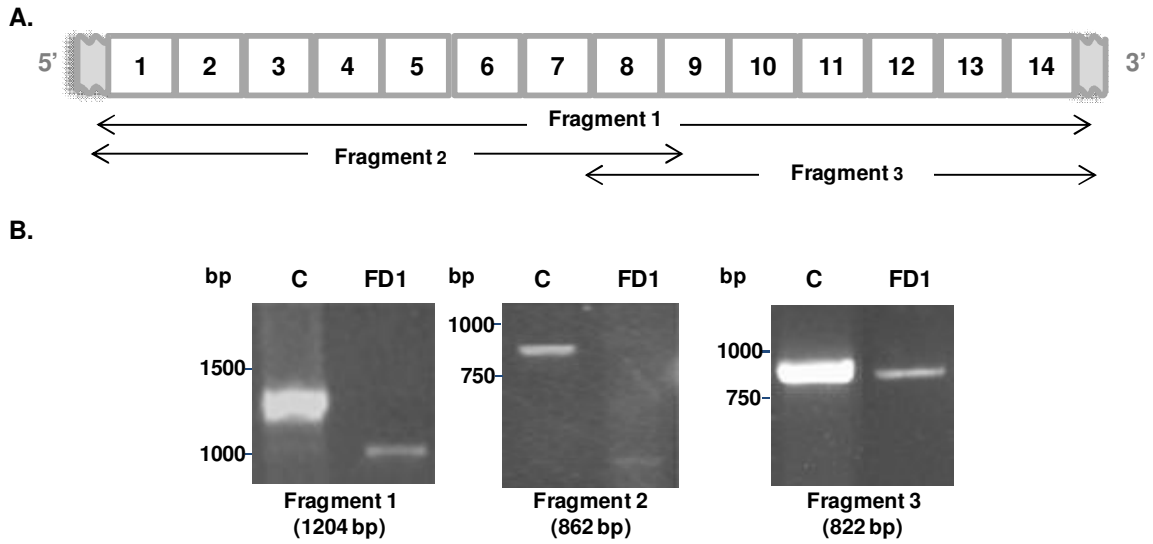

Figure 4.2. RT-PCR products generated from mRNA/*ASAH1*. Total RNA was isolated from control and FD1 patient fibroblasts and reversed transcribed into cDNA as described in the Materials and Methods. **A.** Schematic representation of the amplification strategy of cDNA/*ASAH1* in three overlapping fragments. **B.** Electrophoretic pattern of migration of PCR products. bp, base pairs; C, control; FD1, Farber disease patient 1.

Sequencing data showed that faster migrating bands resulted from the skipping of the full sequence of exons 3 to 5 (c.126_382del) (Figure 4.3.A).This alteration was not detected in 100 normal control chromosomes (data not shown). In addition, several other faster migrating bands were observed (Figure 4.3.B) and sequence analysis revealed that, besides exons 3 to 5, other exonic fragments were also missing, most probably due to alternative splicing that is often over represented when mutations interfere with the cell splicing machinery (Morikawa *et al.,* 2010). In accordance with patient's genotype, the father was heterozygous for the exon-skipping defect and the mother's sequence was normal in this region (data not shown).

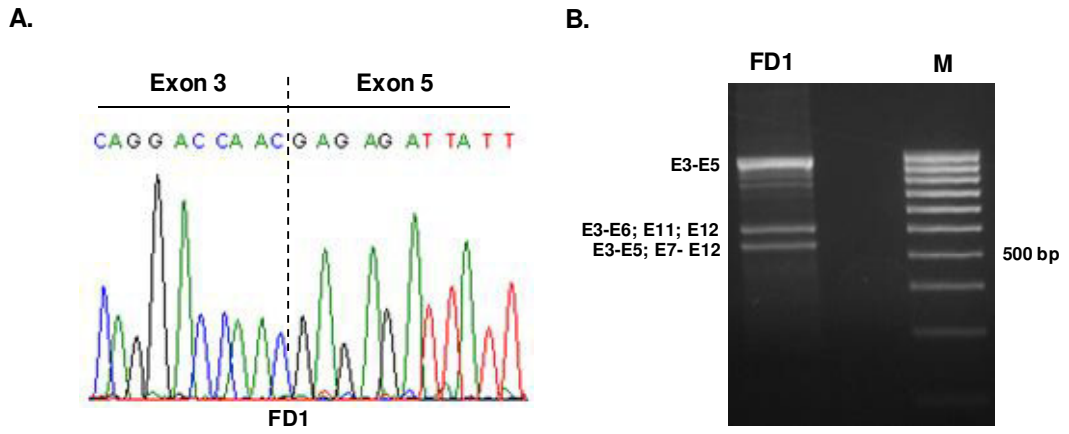

Figure 4.3. Study of cDNA/*ASAH1* PCR products: excision of exons 3 to 5. Total RNA was isolated from control and patient fibroblasts and reversed transcribed into cDNA as described in the Materials and Methods. **A.** Electropherogram obtained after sequencing of PCR products illustrating the deletion of exons 3 to 5 observed in the FD1 patient. **B.** Evidence of alternative splicing in FD patient. Electrophoretic pattern of migration of PCR products generated after the amplification of the full length cDNA/*ASAH1* (fragment 1) is shown. For the most prominent cDNA-PCR bands, DNA was purified and subjected to sequence analysis. Depleted exons are indicated in the left side of the gel. The length of mutant cDNA PCR products observed after agarose gel electrophoresis matched the theoretical prediction. bp, base pairs; C, control; E, Exon; F1, Fragment 1; FD1, Farber disease patient 1; M, DNA molecular weight marker.

IV.1.1.3. Identification of a large *ASAH1* gene deletion

As the patient was heteroallelic for the g.24491A>G mutation and cDNA analysis revealed a fragment lacking the full-sequences of exons 3 to 5, the existence of a large deletion in the *ASAH1* gene was then postulated. To investigate this hypothesis, a long-range PCR was designed to amplify *ASAH1* genomic DNA encompassing exons 2 to 6. After agarose gel electrophoresis, two fragments were observed in patient's sample: the normal amplicon and a shorter fragment that was over-represented probably due to the more efficient amplification of shorter fragments often observed in long PCR analysis (Figure 4.4). Direct sequencing revealed a deletion of 9470 bp (g.8728_18197del), from 3941 nucleotides before the first nucleotide of exon 3 to 1358 nucleotides after the last nucleotide of exon 5 (c.126-3941_382+1358del).

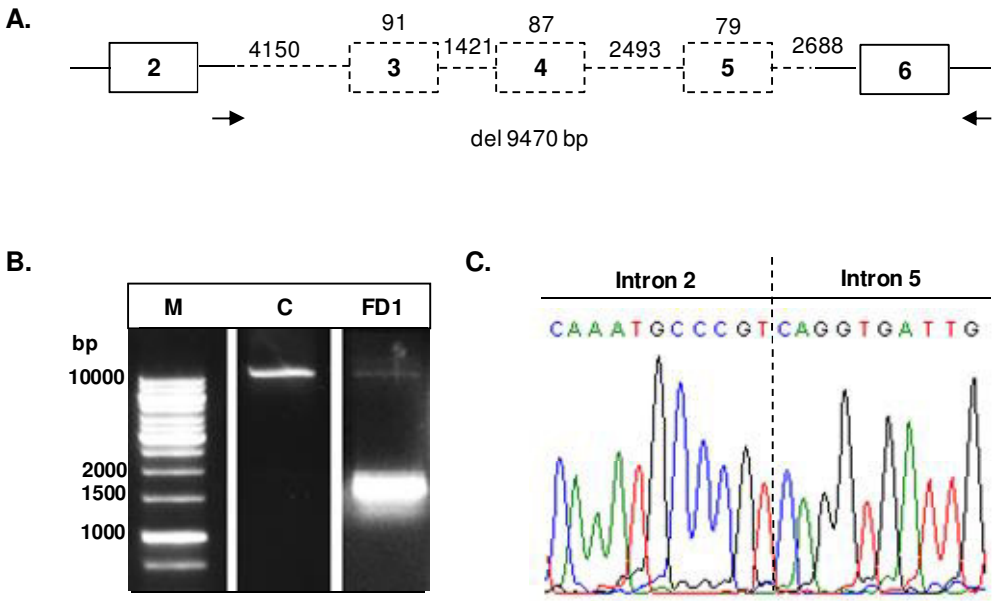

Figure 4.4. Characterization of exons 3 to 5 deletion in *ASAH1* gene. Cycle sequencing of the PCR products obtained from genomic DNA of patient's cells was performed as described in the Materials and Methods. **A.** Genomic region encompassing exons 3 to 5 (represented as boxes) of *ASAH1* gene. The length of each intron and exon is indicated in base pairs. Long-range PCR was performed using a forward primer in the beginning of intron 2 and a reverse primer in intron 6 (represented by arrows). **B.** The amplification products were visualized in agarose gel showing a ~11 kb fragment in control and patient samples and a much more intense PCR product of about 1.8 kb in the patient. **C.** Sequencing analysis encompassing the deletion junction c.126-3941_382+1358del. bp, base pairs; C, control; FD1, Farber disease patient 1; M, DNA molecular weight marker.

The exact breakpoint could not be unambiguously ascertained because of the presence of an identical nucleotide at both ends. The most 3' position (in the sense strand) was used to describe the position of the breakpoint. The deletion event generated a cleaned junction with no missing or inserted nucleotides. Large deletions frequently originate from recombination events promoted by repetitive elements present in the human genome (Callinan and Batzer, 2006), and breakpoints may also occur at predicted non-B DNA structures (rev. in Bacolla and Wells, 2009) Therefore, the DNA sequence flanking the 5' and 3' deletion breakpoints was screened for the presence of such elements or structures. Analysis with Repeatmasker software revealed no sequence homology for interspersed repeat elements across or near the deletion breakpoints. However, these elements are over-represented in introns 2 to 5 of the gene when compared with the entire gene sequence. Sequence analysis with nBMST program identified an inverted repeat across the 3' deletion breakpoint and a stretch of 14 dinucleotide repeats $(AC)_n$ located 23 nucleotides upstream of the 3' breakpoint. Nevertheless, no significant homology was noticed for the intronic sequences in the regions flanking the deletion.

IV.1.1.4. Polymorphic genetic variants

The information on gene polymorphisms may be important in future studies aiming to explore the importance of the backgrounds in which mutations occur. In this regard, two documented single nucleotide polymorphisms were found in FD1 sample: c.457+45A>G in intron 6 (SNP: rs2073574) and c.737T>C in exon 10 (SNP: rs10103355) (Figure 4.5).

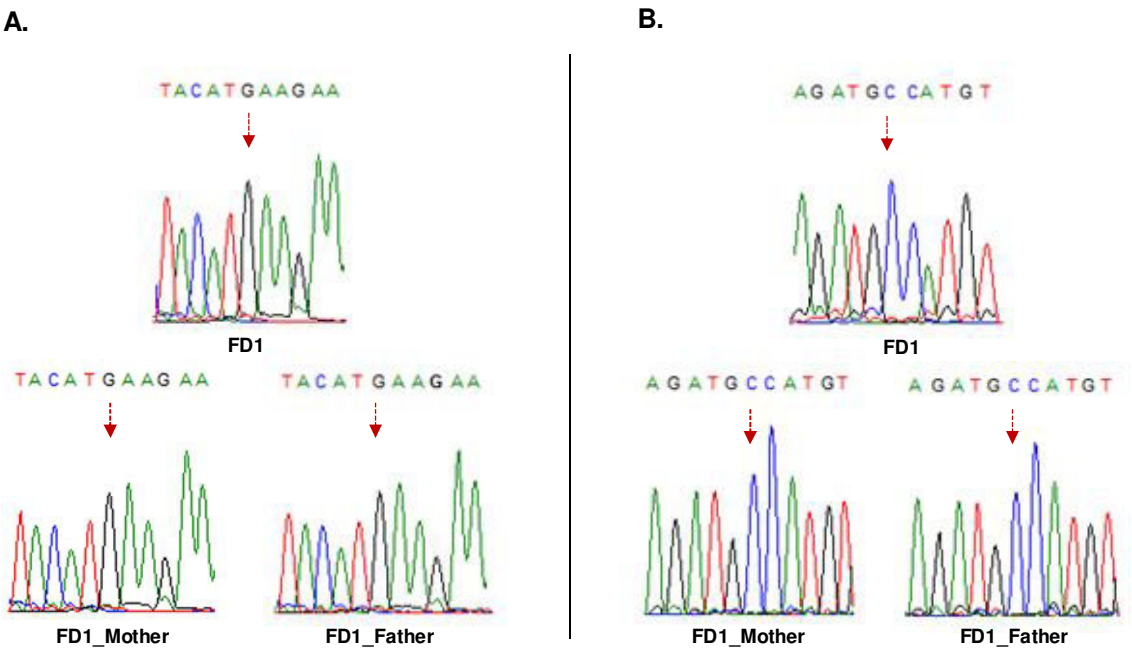

Figure 4.5. Single nucleotide polymorphisms in *ASAH1* gene. The DNA was isolated from patients and parents fibroblasts and analyzed by direct cycle sequencing of PCR products as described in the Material and Methods. Primers are described in Table 3.2. The corresponding positions of SNPs in the electropherograms are indicated by arrows. **A.** Intron 6, c.457+45 A>G (SNP:rs2073574); **B.** Exon 10, c.737 T>C (SNP: rs10103355). FD1, Farber disease patient 1.

In addition, a duplicational insertion of 29-bp in intron 5 was found in homozygosity, c.382+118_382+119dup29 (or c.382+118insGCTGTATGTCTTA GATATACAGCACAG GT) (Figure 4.6.). Several polymorphisms are documented in the region c.382+83-118 (SNP: rs144918941; rs115973081; rs61430183; rs138234399; rs117637498; rs146761887; rs144722013; rs117355478; rs34633536). This observation highlights the highly variable nature of the sequence nearby the mutation. Indeed, the detection of the genetic alteration in 78 of the 100 control chromosomes (data not shown) reinforces the assumption of a polymorphic variant. As the insertion point is flanked by direct repeats, the DNA slippage model might explain the duplication observed (Figure 4.6). This model

assumes that, during the process of DNA replication, the primer strand transiently dissociates from the template strand, the newly synthesized second repeat slips backward to mispair at the first repeat and the progression of the DNA synthesis results in sequence duplication (rev. in Cooper and Krawczak, 1991; Oron-Karni *et al.,* 1997).

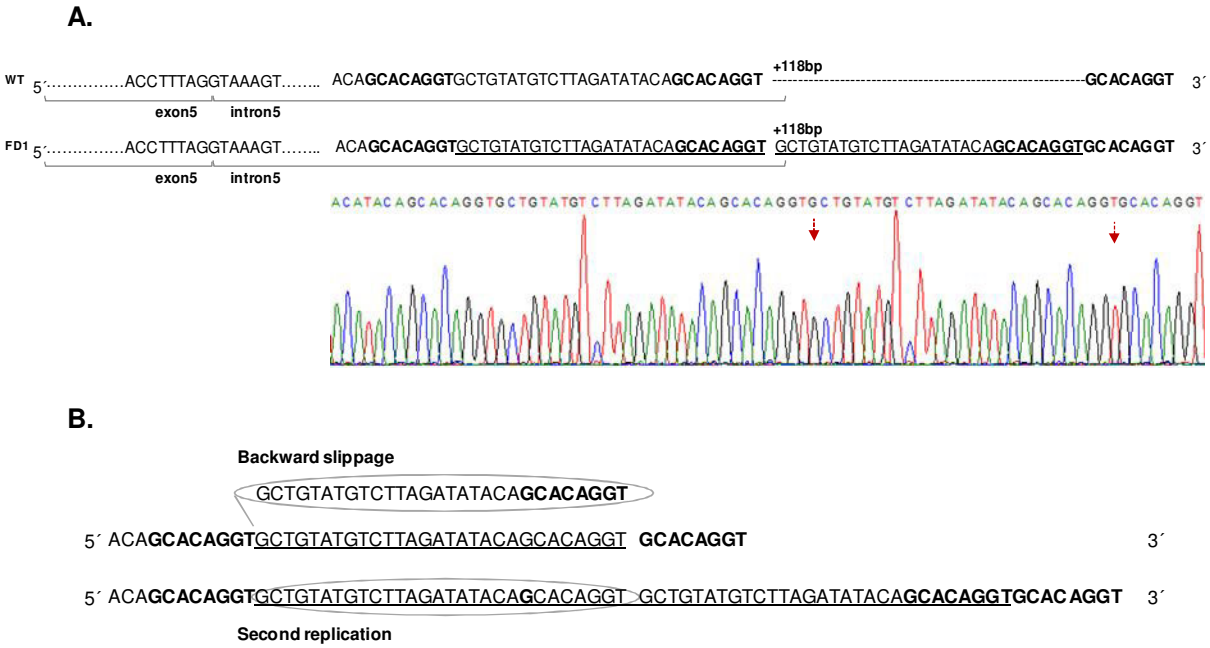

Figure 4.6. Characterization of the duplicational insertion in intron 5 of *ASAH1* gene. A. PCR and cycle sequencing procedure were performed as described in the Material and Methods. The DNA sequence at the mutation breakpoint is underlined and the corresponding position in the electropherogram indicated by an arrow. **B.** Slipped strand mispairing mechanism for the polymorphic duplicational insertion observed in intron 5 of the *ASAH1* gene of FD1 patient. FD1, Farber disease patient 1; WT, Normal wild-type sequence.

The patient's genotype was confirmed by studying parent's samples. The two single nucleotide polymorphisms c.457+45A>G in intron 6, and c.737T>C (p.Val246Ala) in exon 1, were observed in homozygosity in the DNA samples of both parents (Figure 4.5). The insertion of 29-bp in intron 5, observed in a hotspot region of the gene, was transmitted by the mother (data not shown).

IV.2. CHARACTERIZATION OF THE MUTATIONAL MOLECULAR MECHANISMS

To study the biological impact of the mutations identified in FD1 patient, *in silico* prediction studies and analysis at the level of mRNA and protein was then performed.

IV.2.1. Predictions from *in silico* analysis

Since g.24491A>G is a novel mutation, functional studies would be needed to ascertain its pathogenicity. Thus, *in silico* analysis of this mutation was performed. In exon 11/intron 11 splice junction two possible donor splice sites crossing the mutation were recognized by the web tools SpliceView, Human Splicing Finder and MaxEnt model. The higher the score, the higher is the probability that the sequence is a true splice site. Considering that the highest scored sequence, TGAgtaagt, is the true splice site, then the mutation g.24491A>G occurs 4 nucleotides downstream of the first position of intron 11. Ideal MaxEnt splice site score for 5'ss is 11.81. For the mutation g.24491A>G, MaxEnt model estimated the reduction of the 5´ss strength from 8.82 to 3.27 (average score for the donor splice site in the *ASAH1* gene: 8.83). Furthermore, in addition to the splice site, intronic and exonic cis-elements play also an important role in splicing. For the mutation g.24491A>G in intron 11, Human Splicing Finder software predicts the disruption of one exonic splicing enhancer motif for the protein SRp55, and the creation of one exonic splicing enhancer motif for the protein SRp40. Moreover, the creation of one potential splicing silencer motif ([T/G]G[T/A]GGGG) in two positions of the splicing junction sequence, and the creation of three potential exonic splicing silencers-hexamers upstream of the transition were also noticed. These findings further support the disruption of the normal splicing process by the mutation g.24491A>G.

IV.2.2. Impact of mutations on the mRNA and protein levels

IV.2.2.1. Semi-quantitative analysis of the *ASAH1* transcript

Analysis of cDNA-PCR products, using *GAPDH* as endogenous gene for expression normalization, showed a level reduced to about 50% in FD cells when compared with the

control sample (Figure 4.7). This observation suggests that g.24491A>G is likely to have a negligible impact on the level of the total transcript. This notion is also supported by the absence of a detectable band corresponding to the full length cDNA/*ASAH1* in FD sample (Figure 4.2.A, fragment 1). The major abnormal transcript observed in patient's cells is predicted to code for a shortened polypeptide resulting from the deletion of amino acids coded by exons 3 to 5 and the introduction of a premature stop codon due to a frameshift abnormality, p.Tyr42_Leu127delinsArgfs*10.

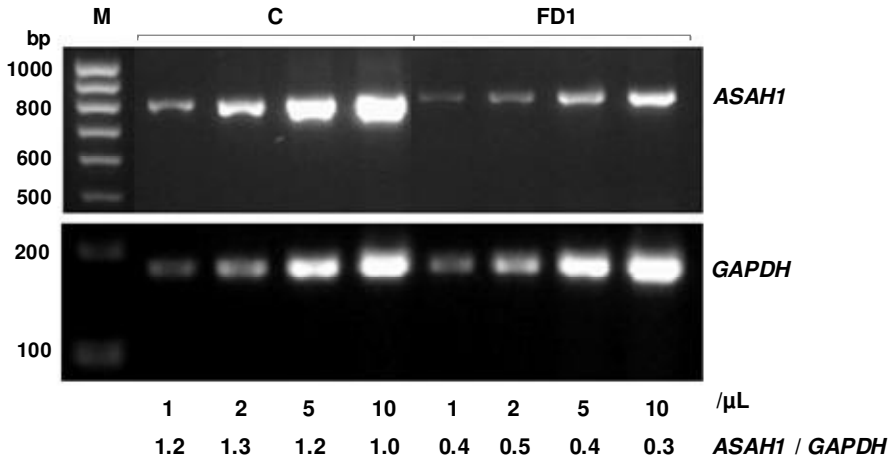

Figure 4.7. Semi-quantitative analysis of cDNA/*ASAH1*. Total RNA was reversed transcribed and used for the amplification of cDNA/*ASAH1* (fragment 3). For expression normalization, *GAPDH* was used as endogenous gene and the RT-PCR exponential phase was considered at 25 cycles for both genes. The expression level referred to as the ratio of *ASAH1*/*GAPDH* is shown below each lane. *ASAH1,* Acid ceramidase gene*;* bp, base pairs; C, control; FD1, Farber disease patient 1; *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase gene; M, DNA molecular weight marker.

IV.2.2.2. Protein analysis by Western Blot

The molecular forms of AC present in total cell lysates from FD1, FD1-AC and control fibroblasts were studied by Western blot using distinct antibodies (Figure 4.8). After SDS-PAGE (under reducing conditions, RC) and blotting, a band of about 14 kDa, corresponding to the α-subunit, was detected with MCA and SHA antibodies in control and FD1-AC cells, but not in patient fibroblasts (Figure 4.8.A). Under the same experimental conditions, a band of about 40 kDa was seen with PCA and SHA antibodies in control and FD1-AC cells, but not in FD1 cells (Figure 4.8.B). This band is likely to represent the mature glycosylated form of the β-subunit as it disappeared when samples were analyzed under non-reducing conditions cells (Figure 4.8.C) which, in turn, allowed the detection of an additional band of about 50-55 kDa, presumably corresponding to the precursor form of the enzyme. As expected, both bands of 14 kDa and 40 kDa showed a higher intensity in AC-transduced cells than in control samples.

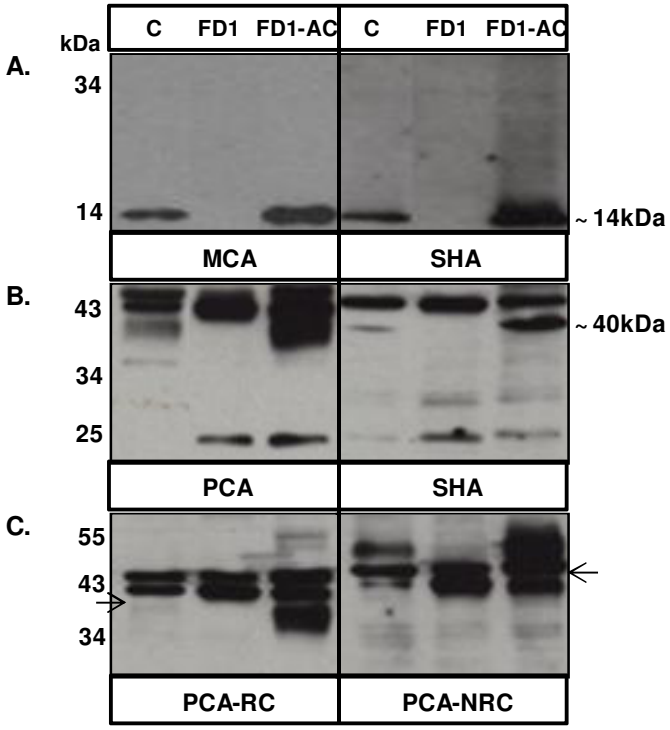

Figure 4.8. Study of acid ceramidase processing in fibroblasts. Fibroblasts lysates were subjected to SDS-PAGE under reducing (Panel A, B and C-RC) or non-reducing conditions (Panel C-NRC), followed by Western blotting as described in Materials and Methods. AC, acid ceramidase; C, control cells; FD1, Farber patient cells; FD1-AC, AC-transduced FD1 cells; kDa, kilodalton; MCA, anti-human AC monoclonal antibody; NRC, non-reducing conditions; PCA, anti-human AC polyclonal antibody; RC, reducing conditions; SHA, anti-recombinant human AC antibody.

IV.3. IDENTIFICATION OF SECONDARY BIOCHEMICAL ALTERATIONS

The FD1 patient is, to our best knowledge, the most severe case of Farber disease ever reported. With the aim to understand if sphingolipid lysosomal hydrolases are altered secondary to the disease and, thus, contribute to the particularly severe phenotype affecting this Farber case, the enzymatic activity of several enzymes was then assessed.

IV.3.1. Enzymatic activity of lysosomal enzymes

A panel of diseased cell lines was used in the study. Besides FD1 and the corresponding cell line transduced with AC cDNA (FD1-AC5x), fibroblasts from three other FD patients, two affected with subtype 1 (FD2 and FD3) and one affected with subtype 3 (FD4), were also included. In addition, NPB cells, which are characterized by the storage of SM due to the deficient enzymatic activity of aSMase, NPC cells, characterized by the accumulation of LDL-derived free cholesterol within LE/lysosomes due to a cellular cholesterol trafficking defect (rev. in Vanier, 2010) and Mucolipidosis type II (MLII) cells, characterized by the deficiency of the enzyme N-acetylglucosamine-1-phosphotranferase (rev. in Lin and Pitukcheewanont, 2012) were also studied.

Firstly, the AC enzymatic activity was reevaluated in this panel of cells (Figure 4.9). In agreement with previous reports (Bär *et al.,* 2001; Kattner *et al.,* 1997; Koch *et al.,* 1996; Li *et al.,* 1999; Medin *et al.,* 1999), the AC enzymatic activity observed in the cells of all FD patients was clearly reduced when compared with control cells (Figure 4.9.A), and the overexpression of a functionally active AC was confirmed in transduced FD1 cells (Figure 4.9.B). Apparently, no correlation was observed between the *in vitro* AC enzymatic activity and the disease's severity. Indeed, the enzyme activity of FD2 and FD3 cells was found slightly increased than that observed in the milder form, FD4 (Figure 4.9.A). Of note, in NPB fibroblasts the enzyme activity was found to be reduced to about 60% of the control mean value (Figure 4.9.A).

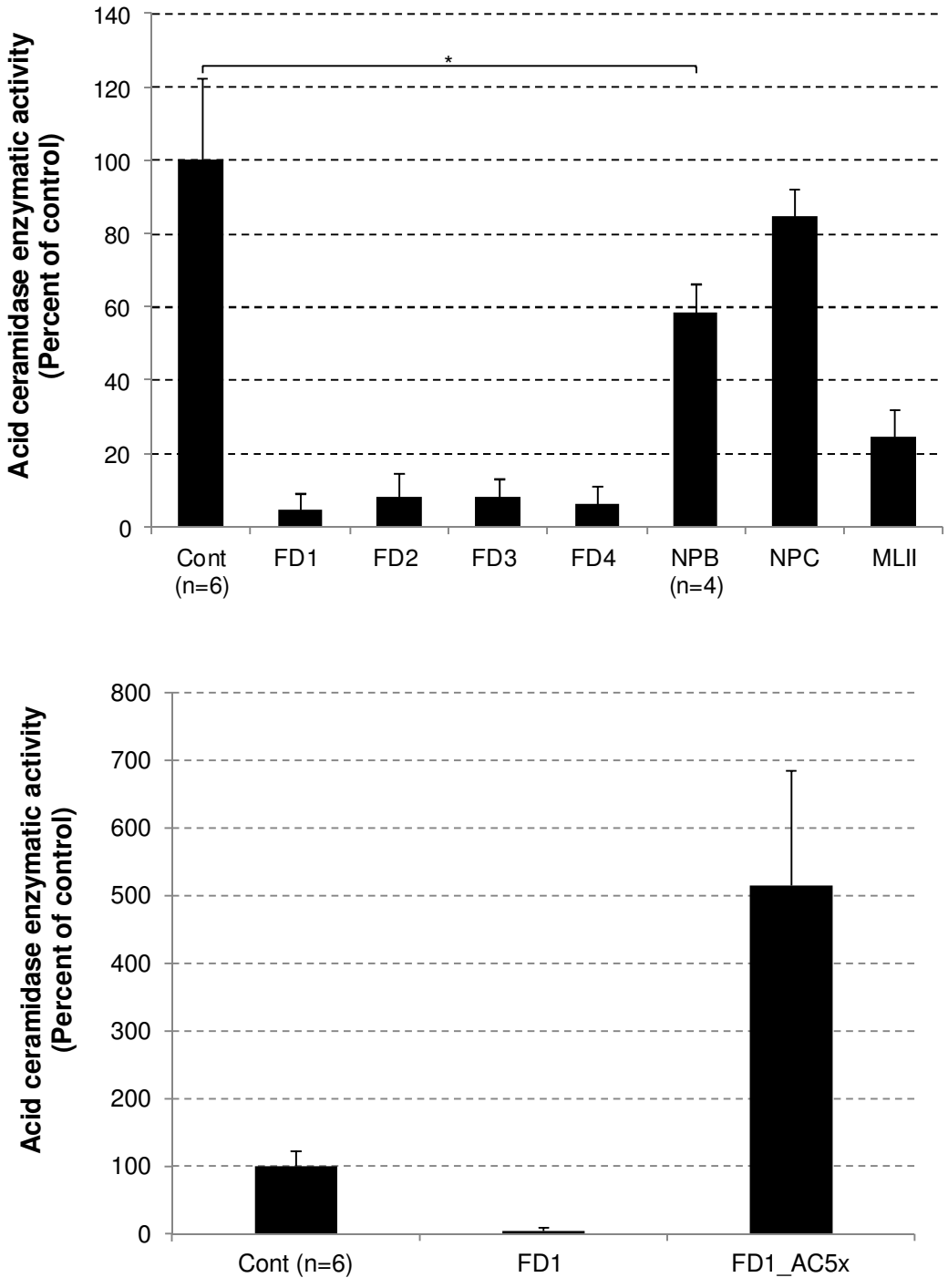

Figure 4.9. Acid ceramidase enzymatic activity in fibroblasts. Total cell extracts were obtained and enzymatic activity determined as described in Materials and Methods. Each assay was performed in duplicate. At least 3 independent determinations were performed for each cell line. In parentheses is shown the number (n) of cell lines included in the study. Data is expressed as percent of control mean value ± SD. Control values (mean ± SD) were 3376 ± 755 nmol/h/mg protein. Similar values were obtained in pSV40 transformed normal fibroblasts. Cont, control fibroblasts; FD, Farber patients's fibroblasts; FD-AC5x, AC-transduced Farber fibroblasts; MLII, Mucolipidosis type II fibroblasts; NPB, Niemann-Pick type B fibroblasts; NPC, Niemann-Pick type C fibroblasts. *p-value≤0.005.

The enzymatic activity of aSMase (EC 3.1.4.12), β-galactocerebrosidase (EC 3.2.1.46, GalCase), β-glucocerebrosidase (EC 3.2.1.45, GlcCase), total β-hexosaminidase (EC 3.2.1.52, HexT) and β-galactosidase (E.C 3.2.1.23, Glase) was then studied in FD1 cells through specific *in vitro* assays using fluorogenic synthetic substrates (Table 4.1).

Table 4.1. Enzymatic activity of lysosomal enzymes in fibroblast cell lysates.

	AC	aSMase	GalCer	GlcCer	HexT	Glase
FD	0.15 ± 0.16 (6)	14 ± 8 (6)	1.0 ± 0.2 (6)	83 ± 18 (6)	3574 ± 800 (6)	368 ± 68 (6)
NPB	2.0 ± 0.3 (n=4)	1.5 ± 1.0 (n=4)	ND	ND	ND	ND
NPC	2.9 ± 0.3 (3)	12 ± 4 (3)	ND	ND	ND	ND
Control	3.4 ± 0.8 (n=6)	68 ± 19 (n=18)	1.2 ± 0.4 (n=6)	197 ± 39 (n=6)	6255 ± 926 (n=6)	760 ± 127 (n=6)

The enzymatic activities were determined against synthetic substrates has described in Materials and Methods. The enzymatic activity (nmol/h/mg protein) is expressed as mean ± SD. Each assay was performed in duplicate; in parentheses is shown the number of separate determinations or the number (n) of cell lines included in the study. AC, Acid ceramidase; aSMase, Acid sphingomyelinase; FD, Farber patient's fibroblasts; Glase, β-galactosidase; GalCase, β-galactocerebrosidase; GlcCase, β-glucocerebrosidase; HexT, total β-hexosaminidase; ND, Not determined; NPB, Niemann-Pick type B fibroblasts; NPC, Niemann-Pick type C fibroblasts.

With the exception of the enzymatic activity of aSMase that corresponded to about 20% of the average control value, the activity of the other lysosomal enzymes was found within the control range values, although for GlcCase, HexT and Glase a decreased of about 50% in comparison with the average control value was observed.

The *in vitro* enzymatic activity of aSMase was next evaluated in the panel of diseased cells included in the study using the natural radioactive substrate (choline-methyl-^{14}C sphingomyelin) (Figure 4.10).

The enzymatic activity of aSMase was found surprisingly low in FD1 cells and comparable to that observed in MLII cells. It corresponded to about 25% of the average control value, which was slightly higher than observed with the synthetic substrate

reflecting, probably, the higher affinity of the enzyme against the natural substrate. However, a similar reduction was not found in the other FD cells. Moreover, AC overexpression did not correct the enzyme deficiency. As expected, the deficient aSMase enzymatic activity was confirmed in NPB cells. Of note, the residual enzymatic activity of aSMase found in the culture medium of FD1 cells was comparable to the one observed in the culture medium of control cells (data not shown), suggesting that the enzyme produced by FD1 cells is not preferentially secreted.

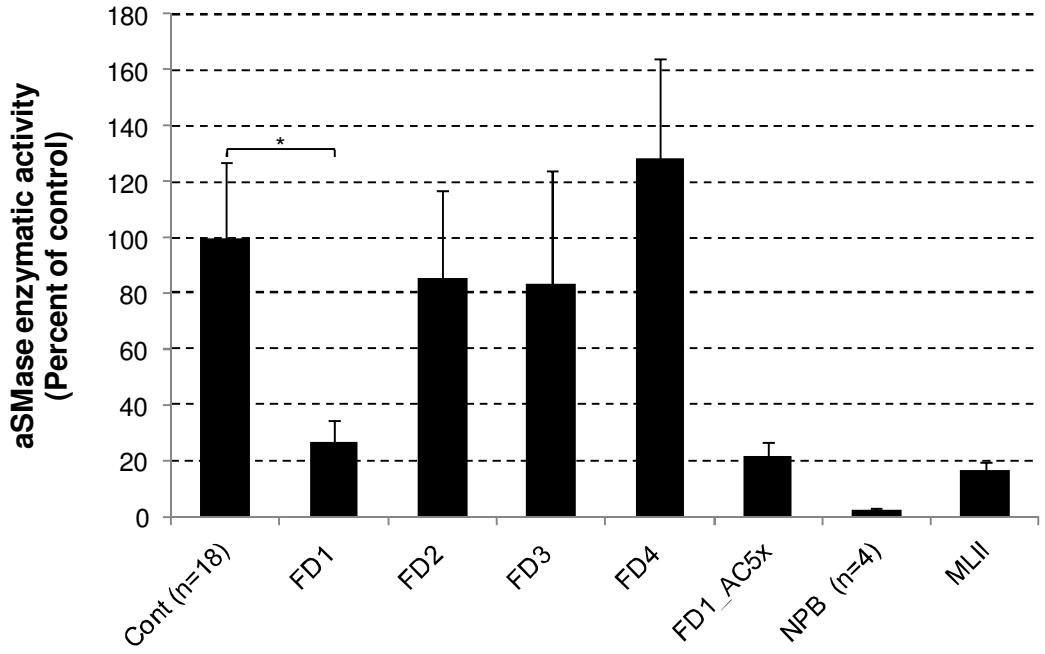

Figure 4.10. Acid sphingomyelinase enzymatic activity in fibroblasts. Total cell extracts were obtained and enzymatic activity determined against choline-methyl-^{14}C sphingomyelin as described in Materials and Methods. Each assay was performed in duplicate. At least 3 independent determinations were performed for each cell line. In parentheses is shown the number (n) of cell lines included in the study. Data is expressed as percent of control mean value ± SD. Control values (mean ± SD) were 107 ± 29 nmol/h/mg protein. Similar values were obtained in pSV40 transformed normal fibroblasts. Cont, control fibroblasts; FD, Farber patients's fibroblasts; FD-AC5x, AC-transduced Farber fibroblasts; MLII, Mucolipidosis type II fibroblasts; NPB, Niemann-Pick type B fibroblasts. *p-value≤0.00001.

As one possible explanation for the partial enzymatic deficiency of aSMase observed in FD1 cells would be the existence of deleterious defects in the gene coding for human aSMase, mutational analysis in *SMPD1* gene was then carried out.

IV.3.2. Mutational screening in *SMPD1* gene

SMPD1 genomic and cDNA from FD1 patient's fibroblasts were analyzed for the presence of mutations. To that end, PCR products corresponding to each of the 6 exons of the *SMPD1* gene and the corresponding intronic flanking sequences were subjected to sequence analysis. In addition, PCR products obtained from the amplification of five overlapping cDNA/*SMPD1* fragments were also sequenced (Figure 4.11). However, no putative pathogenic alterations were found in either *SMPD1* genomic DNA or full length cDNA.

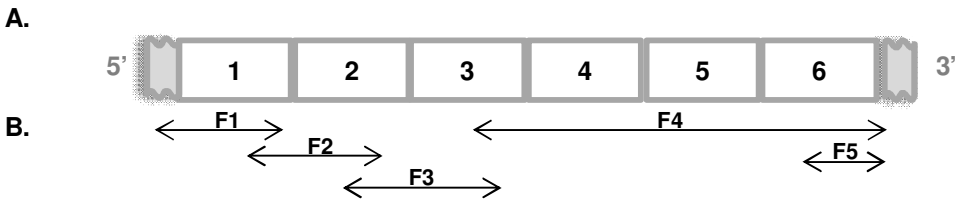

Figure 4.11. Mutational screening in *SMPD1* gene. A. Schematic representation of the *SMPD1* gene. **B.** Amplification strategy of cDNA/*SMPD1* in five overlapping fragments. F, fragment.

We then questioned if the level of mRNA/*SMPD1* would be reduced in FD1 cells. Therefore, semi-quantitative analysis of the transcript was performed (Figure 4.12).

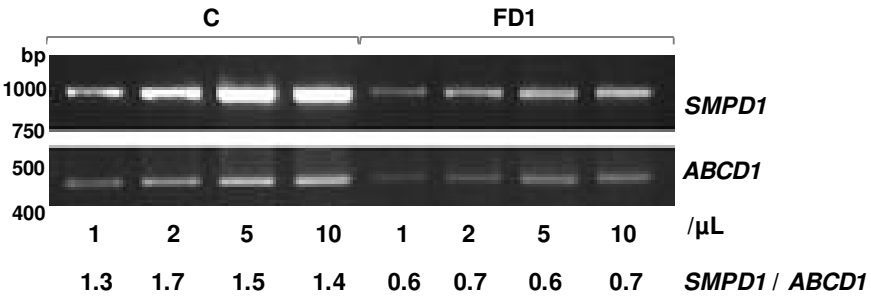

Figure 4.12. Semi-quantitative analysis of cDNA/*SMPD1*. For expression normalization, *ABCD1* was used as endogenous gene and the RT-PCR exponential phase was considered at 25 cycles for both genes. The expression level referred to as the ratio of *SMPD1*/*ABCD1* is shown below each lane. *ABCD1*, Gene coding for adrenoleukodystrophy protein; bp, base pairs; C, Control; FD1, Farber disease patient 1; *SMPD1,* Acid sphingomyelinase gene.

In FD1 patient's cells, a decreased level of mRNA/*SMPD1* of at least 50% when compared with control cells was observed, thus suggesting that the partial enzymatic deficiency observed for the aSMase can be explained, at least in part, by negative modulation of the transcription of the gene. However, its exact mechanism is presently unclear.

In order to understand if the reduced aSMase enzymatic activity observed in FD1 cells would have any impact in the level of total cell SM, the level of two structurally closed and metabolic linked SLs, SM and Cer, was then assessed in fibroblasts.

IV.3.3. Sphingolipid analysis in fibroblasts

In all four FD fibroblast lysates, the level of total Cer and SM was determined (Figure 4.13.A) and data analyzed in terms of the ratio value SM/Cer (Figure 4.13.B). In NPB cells, in addition to the increased level of total SM, a higher level of total Cer compared with the average level in control cells was observed. Of note, the average level of the SM to Cer ratio was only slightly increased when compared to control cells. In FD1 case the increased level of Cer was confirmed and the level of SM found in the lower range of control values. The SM/Cer, which was considerably reduced when compared with control cells, was corrected by the overexpression of AC. In fibroblasts from a milder FD case, FD4, the level of SM approximated that of control cells and the SM/Cer was about 2-fold higher than in the neonatal FD1 case. In FD2 and FD3 cases, corresponding to a lesser severe form of the disease than FD1, the level of Cer was slightly higher than in FD1 cells. These findings are in agreement with the notion that the clinical phenotype is not correlated with the level of stored Cer (Bär *et al.,* 2001; van Echten-Deckert *et al.,* 1997). In NPC cells no alterations were noticed regarding to either Cer and SM levels, or the ratio value (data not shown).

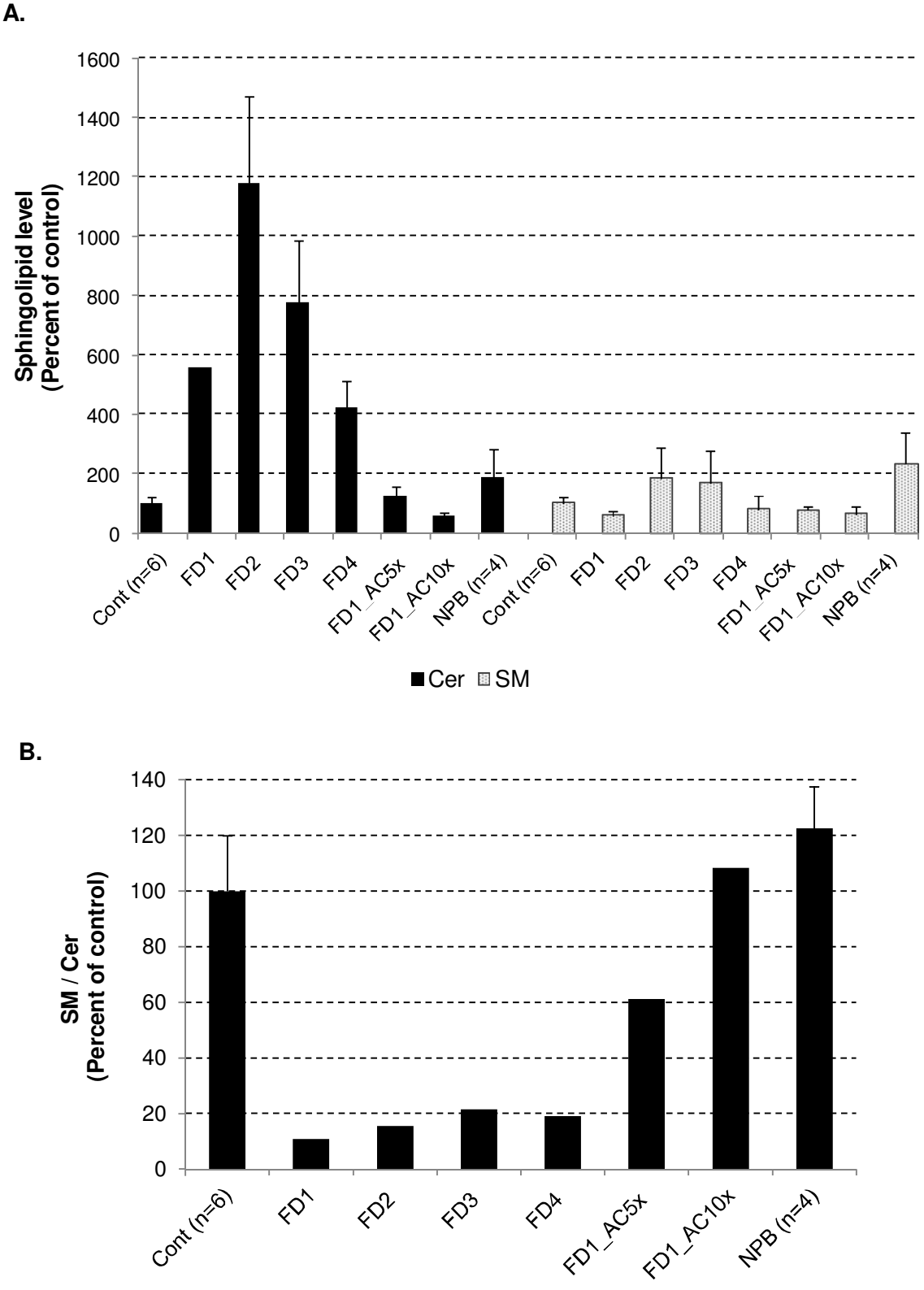

Figure 4.13. Ceramide and sphingomyelin levels in fibroblasts. Lipids were extracted and quantified as described in the Materials and Methods. Each assay was performed in duplicate. At least 3 independent determinations were performed for each cell line. Similar values to control cells were obtained in pSV40 transformed control fibroblasts. Data is expressed as percent of control mean value ± SD. Control values for ceramide (**A**), sphingomyelin (**A**) and SM/Cer ratio (**B**) were 1350 ± 292 pmol/mg, 26900 ± 5859 pmol/mg and 19.4 ± 4.0, respectively. Cer, Ceramide; Cont, control fibroblasts; FD, Farber patients's fibroblasts; FD-AC, AC-transduced FD fibroblasts (5x- or 10x-infected cells); NPB, Niemann-Pick type B fibroblasts; SM, Sphingomyelin.

Based on the fact that neither the amount of accumulated Cer nor residual AC activity in FD fibroblasts was apparently correlated with the disease onset and progression, and in cells from FD1 patient, representing the most severe form of the disease, significant alterations were found for either the enzymatic activity of aSMase or the ratio value of total Cer for total SM, the relation between these two variables were then analyzed (Figure 4.14).

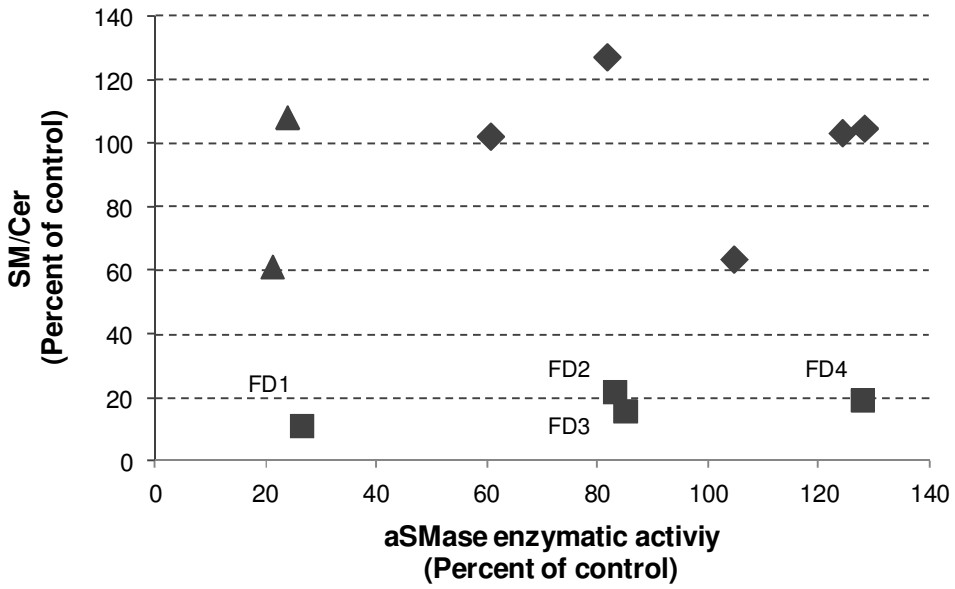

Figure 4.14. Analysis of the ratio of total cellular sphingomyelin and ceramide *vs.* aSMase enzymatic activity. Fibroblast cells were used for lipids analysis and enzymatic assays according to the procedures described in Materials and Methods. For each case, the mean value was referred as percentage of the control mean value. aSMase, Acid sphingomyelinase; Cer, Ceramide; Control cells (♦); FD, Farber patients's cells (■); FD-AC, AC-transduced FD cells (5x- or 10x-infected cells, ▲). For each case, the mean value was referred as percentage of the control mean value.

The ratio value SM/Cer clearly discriminated controls from FD cells, whereas the enzymatic activity of aSMase allowed, apparently, the differentiation between the neonatal (FD1 case), subtype 1 (FD2 and FD3 cases) and subtype 3 (FD4 cases) phenotypes.

Chapter V

General Discussion and Future Perspectives

Farber disease or Farber's lipogranulomatosis (FD, MIM 228000) is a recessively inherited sphingolipid (SL) storage disorder caused by the deficiency of AC that leads to the intralysosomal accumulation of ceramide. Typical symptoms, generally noticed early in life, include the unique triad of subcutaneous nodules, painful and progressively deformed joints, and hoarseness by laryngeal involvement. Based on the age of onset, the severity of symptoms, and the difference in organ affection by ceramide storage, seven disease subtypes are distinguished (Moser *et al.,* 2001). Subtypes 1 to 6 are associated with the deficiency of AC caused by mutations in the *ASAH1* gene, whereas subtype 7 is caused by the deficiency of the SAP precursor, prosaposin. Except for subtype 7, the most severe form is subtype 4, a rare neonatal form of the disease described to date in only about 7 patients (Antonarakis *et al.,* 1984; Qualman *et al.,* 1987; Nowaczyk *et al.,* 1996; Willis et al., 2008).

Although 80 patients with the diagnosis of FD are referred in the literature (Levade *et al.,* 2009; Moser *et al.,* 2001), the genotype was characterized thus far in only 20 patients leading to the identification of 23 mutations in the *ASAH1* gene, mostly missense mutations (17/23) and associated with the subtypes 1 and 3 of the disease (Al Jasmin, 2012; Bär *et al.,* 2001; Chedrawi *et al.,* 2012; Cvitanovic-Sojat *et al.,* 2011; Devi *et al.,* 2006; Koch *et al.,*1996; Kostik *et al.,* 2013; Levade *et al.,* 2009; Li *et al.,* 1999; Muramatsu *et al.,* 2002; Muranjan *et al.,* 2011; Zhang *et al.,* 2000). Thus, comparing with other lysosomal diseases few causative mutations of FD are known, and for the vast majority of them the molecular mechanism is unclear. More recently, mutations in this gene have also been reported in patients with spinal muscular atrophy with progressive myoclonic epilepsy (MIM 159950) (Zhou *et al.,* 2012), suggesting that Farber disease may be widely underestimated. Aiming to contribute to extend the information in this particular field, cultured skin fibroblasts from a neonatal FD patient presenting the most severe clinical manifestations of AC-deficiency ever described (Kattner *et al.,* 1997; Schäfer *et al.,* 1996) were studied by integrating data from DNA, RNA, protein and SL analysis.

V.1. ELUCIDATION OF THE PRIMARY DEFECTS IN THE NEONATAL FORM OF FARBER DISEASE

In this work, the genotyping of the first, and to our knowledge the only, reported FD case with non-immune hydrops revealed a compound heterozygous carrying two novel mutations: g.24491A>G (c.917+4A>G), in intron 11 and the large deletion g.8728_18197del (c.126-3941_382+1358del), encompassing exons 3 to 5 of *ASAH1* gene. The transition in the 5'ss consensus sequence of intron 11 represents the third report of a splicing mutation in the *ASAH1* gene (c.457+4A>G associated with the disease's subtype 1 and 5 (Muranjan *et al.,* 2011), c.1098+1G>T with subtype 5 (Bär *et al.,* 2001) and present study). The second mutation is the first, and to our best knowledge the only, gross deletion reported in the *ASAH1* gene.

Based on data reported herein, FD cases with mutation(s) detected in only one allele on the conventional analysis of just *ASAH1* genomic DNA must be examined for the presence of a large heterozygous deletion in the second allele. Without direct cDNA analysis, this kind of mutations can remain unidentified. In the literature, at least 3 FD patients represent possible candidates for the presence of a large deletion in the second allele (Al Jasmi, 2011; Bär *et al.,* 2001; Muranjan *et al.,* 2011).

The precise ascertainment of the genotype is important for a correct establishment of genotype-phenotype correlations and, subsequently, for genetic counseling of patients and families with this specific rare disease. In this regard, experimental and *in silico* data analysis presented in this study clearly demonstrate that these two novel mutations result in null alleles, thus explaining the disease's clinical severity of this compound heterozygous FD case. The primary transcript generated by the allele carrying the splicing mutation is likely to be prematurely degraded as no detectable transcripts were seen after cDNA amplification. For the other allele, the main identified transcript lacked the entire sequences of exons 3 to 5, predicting the production of a truncated polypeptide with only 52 amino acids, of which 42 correspond to the wild-type N-terminal primary sequence of AC. No detectable molecular forms corresponding to precursor or proteolytically processed mature protein were observed. Thus, if this aberrant transcript escapes to degradation by the nonsense-mediated mRNA decay mechanism (Chang *et al.,* 2007) and is translated, then the mutant polypeptide must be prematurely degraded. In compliance with these findings, the *in vitro* residual enzymatic activity observed in patient's cells (Kattner *et al.,* 1997) represents one of the lowest values reported for FD patients (Levade *et al.,* 2009).

The presence of two loss-of-function mutations in the rare neonatal form of FD, as demonstrated in this work, indicates that the last step of the SL degradation pathway must be deeply blocked due to the absence of any functionally active AC. Therefore, patient's cells represent an attractive cellular model to explore downstream effects of lysosomal AC-deficiency, especially if considering that the murine knock-out model was found not viable (Li *et al*., 2002). However, very recently, after the conclusion of the experimental part of the work described in this thesis, the first viable animal model of systemic AC deficiency was reported (Alayoubi *et al.,* 2013). This mouse model harboring a single nucleotide mutation in *ASAH1* gene showed characteristic features of FD, including growth retardation and early death, reduced enzyme activities, accumulation of ceramide, abnormal infiltrations with foamy macrophages in various tissues, and the presence of Farber bodies by electron microscopy. Furthermore, lentivector-mediated neonatal gene delivery of human *ASAH1* / cDNA partially corrected the disease manifestations. This new animal model of AC deficiency is particularly valuable not only to future studies on pathophysiology of FD, namely, the effects of ceramide storage in different tissues and organ environments, but also to study SLs metabolism.

V.2. IDENTIFICATION OF SECONDARY BIOCHEMICAL ALTERATIONS

In all cells of FD patients enrolled in this study, the deficient enzymatic activity of AC was confirmed. Moreover, in cells overexpressing AC a substantially increased of the enzymatic activity was observed, as previously reported (Medin *et al.,* 1999).

Previous studies suggested the importance of a balanced level of several lysosomal enzymes in the maintenance of cellular ceramide levels, namely various sphingomyelinases and ceramidases (Ferlinz *et al.,* 2001), and also the possibility that AC could be part of a multienzymatic complex, integrating acid sphingomyelinase (aSMase) and β-galactosidase but not several other lysosomal enzymes (He *et al.,* 2003).

The measurement of the *in vitro* enzymatic activity of several lysosomal enzymes in fibroblasts from the neonatal FD case showed that, in addition to the enzymatic deficiency of AC, a decrease of about 25% in the enzymatic activity of aSMase was also observed, while for the other lysosomal enzymes (β-galactocerebrosidase, β-glucocerebrosidase, total β-hexosaminidase and β-galactosidase) the values were comparable to normal cells. As expected, in fibroblasts of patients affected with MLII or NPB, a similar decrease of aSMase enzymatic activity was observed. In MLII, the enzymatic deficiency of N-

acetylglucosamine-1-phosphotranferase prevents the acquisition of the M6P marker by lysosomal soluble proteins resulting in its preferential secretion (rev. in Lin and Pitukcheewanont, 2012). In NPA and NPB the primary defect is the enzymatic deficiency of aSMase caused by mutations in the *SMPD1,* but the enzymatic deficiency is generally more severe in NPA than in NPB. In NPC, most of patients have mutations in *NPC1* gene and mutant cells show reduced ability to esterify cholesterol after loading with exogenously derived LDL-cholesterol. As NPC is characterized by a secondary reduction of aSMase enzymatic activity, all three types are considered forms of the same disease (rev. in Vanier, 2010). Apparently, the reduced aSMase enzymatic activity observed in the neonatal FD cells was not warranted by the presence of putative pathogenic genetic alterations in the gene coding for the human aSMase, the *SMPD1* gene. However, an alteration in the level of mRNA/*SMPD1* to about 50% of control suggests the negative modulation of the *SMPD1* gene at the transcriptional level. The reason for this observation is presently unclear. Recent bioinformatics and functional data propose the existence of the CLEAR gene network and the TFEB as master regulator of lysosomal biogenesis (Sardiello *et al.,* 2009). Interestingly, *ASAH1*, but not *SMPD1*, is a target gene of TFEB (Palmieri M *et al.,* 2011). It is, therefore, possible that transcription of the *SMPD1* gene may be regulated by other transcription factors and/or by a distinct transcriptional mechanism.

In this regard, it is important to underline that the *SMPD1* gene is within an imprinted region of the human genome (Simonaro *et al.,* 2006) and the implication of this observation warrants further investigation in the future. Another interesting finding was the statistically significant decreased of the enzymatic activity of AC in NPB fibroblasts. In this disease the disruption of the SL catabolic pathway is located one step upstream of that catalysed by the AC. The secondary partial enzymatic deficiency observed in FD and NPB, aSMase and AC, respectively, supports the notion that these enzymes may, indeed, interact *in vivo* as previously suggested (He *et al.,* 2003). As consequence, the aSMase downregulation observed in cells from the neonatal FD case may represent a compensatory cell mechanism resulting from the inexistence of any functionally active AC. As a matter of fact, for the other FD cell lines included in this study which represent disease's subtypes 1 and 3, the activity of aSMase was not found particularly reduced in comparison with control cells, but otherwise their clinical phenotype is not as severe as in the neonatal FD case. A similar observation had been previously reported by other authors (van Echten-Deckert *et al.,* 1997) who included in the study fibroblasts of several FD patients, the most severe one with onset at 3 months and death at 6 months. Based in these observations, it would be plausible to consider that the inhibition of aSMase

enzymatic activity by ceramide occurs only when this metabolite attains a substantially high concentration thus, justifying the data obtained in cells from the neonatal FD patient. However, overexpression of AC did not correct for the partial enzymatic deficiency of aSMase observed in non-transduced FD cells. Even so, as the neonatal form here studied represents a unique cell model due to the extensive, or even total, blockage of the last step of the lysosomal SL catabolic pathway, the intracellular level of two structurally close and metabolically linked SLs, Cer and SM, were determined and reanalysed.

As expected, in FD and NPB cells the level of total Cer and SM was significantly increased with respect to control cells, respectively. The AC overexpression corrected the ceramide storage observed in cells from the neonatal form as previously reported (Medin *et al.,* 1999). Moreover, an increased level of total Cer was observed in NPB cells. This observation is in accordance with the partial enzymatic deficiency of AC observed in these cells. In the neonatal FD cells, the enzymatic activity observed for aSMase which corresponded to about 25% of the average control activity, would predict an increased level of total SM. Surprisingly, a reduced level of total cell SM to about 50% was observed when compared with the average level of control cells. In both cases, the difference with respect to control cells was not statistically significant, although it was close to the significance level of 0.05. However, it must be noted that the assay used in lipid quantitation introduced small variations and the state of the cell culture, i.e. degree of cell confluency and passage number, may also have contributed with additional variations.

SL data was also analyzed in terms of the total level of SM *vs.* Cer. This ratio was significantly decreased in all FD cells, particularly in the neonatal form (subtype 4), representing less than 15% of the average control value. However, an inverse proportion, i.e. a significant increase of the ratio SM/Cer, was not seen in NPB cells. Thus, the imbalance of total SM to total Cer observed in FD fibroblasts is likely to be due not only to the abnormally high level of total Cer but also to a slightly decreased level of total SM. Of note, the overexpression of AC normalized the ratio observed for the total level of SM and Cer, strongly suggesting that specific steps of the complex network of metabolic reactions located downstream of lysosomal sphingosine (Sph) are under tight regulation. As a matter of fact, in the neonatal FD cells, the level of lysosomal Sph is likely to be dramatically unbalanced, conditioning the downstream synthesis of Cer and therefore the level of Cer (from the salvage pathway) that is returned to the cell metabolism, namely for the SM synthesis at the GC. This would explain, at least in part, why the level of total SM is reduced in fibroblasts from neonatal FD patient.

As already mentioned above, in NPA and NPB, the disruption of the SL catabolic pathway is located one step upstream of that catalysed by the AC. Altogether, these findings suggest that a yet unknown balancing mechanism(s) is altered in NPB fibroblasts and, apparently, blocked in FD. A deep unbalanced level of Cer to SM, as observed in FD fibroblasts, would contribute to the alteration of biophysical properties of membranes and explain the disease symptoms. Therefore, the impairment of AC enzymatic activity is likely to be more dramatic for the cell to keep the balance level of the two bioactive SLs, Cer and SM, than the deficiency in the enzymatic activity of aSMase, thus justifying the disease's severity of the Farber case. As the steady state level of cell Cer and SM result from a complex network of reactions in which AC and aSMase also participate, further studies must be carried out to evaluate other metabolic points of this intricate cell network.

In agreement with other studies (van Echten-Deckert *et al.,* 1997), data here report support the notion that the severity of the FD clinical phenotype is neither correlated with the AC residual enzymatic activity nor the level of total Cer. Nevertheless, some controversy still exists regarding to this issue (Chatelut *et al.,* 1996; van Echten-Deckert *et al.,* 1997; Levade *et al.,* 1995b; present study). Therefore, data reported herein was also analyzed in terms of the ratio value SM/Cer *vs.* the enzymatic activity of aSMase. Controls were clearly distinguished by FD patients through the ratio value of SM/Cer, and the clinical phenotypes of the FD patients enrolled in this study, i.e. subtypes 4, 1 and 3, were, apparently, discriminated by the enzymatic activity of aSMase. This preliminary data suggest that aSMase can be regarded as a potential disease biomarker. However, the number of patients enrolled in this study was too small to draw definitive conclusions and, therefore, this analysis must be extended, in future, to more FD patients.

Finally, in view of the findings here reported, an accurate assessment of the level of SLs seems to be helpful in detecting secondary imbalances in the cellular content and distribution of these lipid molecules, including GSLs, which might be informative for the disease progression. Moreover, it is also important to note that as FD is considered a very rare storage disorder, the enzymatic assay of AC may be excluded from the panel of biochemical enzymatic assays used in the diagnosis of LSDs. Moreover, typical clinical signs of the disease may not be present in neonatal forms. In this regard, this work highlights the importance to consider the biochemical study of FD in those cases presenting a decreased aSMase enzymatic activity but in which Niemann-Pick disease (types A, B and C) and MLII have been biochemically ruled out.

In conclusion, in this work the mutations underlying the neonatal form of FD were described for the first time, two novel null alleles identified and the first gross deletion in

the *ASAH1* gene reported, which not only broad the spectrum of disease-causing mutations and enlarge the knowledge on the molecular mechanisms of FD, but they also contributed for the accuracy of disease's molecular diagnosis. Additionally, data described herein also give strength to the hypothesis that AC and aSMase may, indeed, interact *in vivo* and report for the first time alterations in the total level of Cer *vs.* SM that are likely to have important pathophysiological implications to FD. Nevertheless, a more profound characterization of the basic properties of AC, the nature of AC-aSMase interaction, and other possible interaction partners, and the downstream consequences of the AC deficiency, namely at the level and cell distribution of SL and GSL molecules, represent key points that must be addressed in future works to improve our understanding about AC cell biology in normal and disease conditions.

Chapter VI

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Web Resources

The URLs for data presented herein as follows:

Ensembl Genome Browser, http://www.ensembl.org/

Genetics Home reference, http://ghr.nlm.nih.gov/

HUGO Gene Nomenclature Committee (HGNC), http://www.genenames.org/

Human Gene Mutation Database website (HGMD), http://www.hgmd.org

Human Genome Variation Society (HGVS), http://www.hgvs.org/mutnomen/

Human Splicing Finder (HSF), http://www.umd.be/HSF/

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

Scriver's OMMBID: The Online Metabolic and Molecular Bases of Inherited Diseases, edited by D. Valle et al, 2012. http://www.ommbid.com/

Appendix

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Molecular basis of acid ceramidase deficiency in a neonatal form of Farber disease: Identification of the first large deletion in *ASAH1* gene

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ABSTRACT

Farber disease, also known as Farber's lipogranulomatosis, is a clinically heterogeneous autosomal recessive disease caused by mutations in the *ASAH1* gene. This gene codes for acid ceramidase, a lysosomal heterodimeric enzyme that hydrolyzes ceramide into sphingosine and fatty acid. To date, less than 25 distinct mutations have been identified in Farber patients, but no large deletions have yet been reported. In this work, cultured fibroblasts from a Farber patient with the rare neonatal form of Farber disease were studied to elucidate the molecular basis of this extremely severe phenotype. Direct sequencing of *ASAH1* genomic DNA revealed the causative heterozygous mutation in the donor splice site consensus sequence of intron 11, g.24491A > G (c.917 + 4A > G), that resulted in the absence of detectable mRNA. Subsequent analysis of *ASAH1* mRNA showed total skipping of exons 3 to 5. Long-range PCR and sequencing led to the identification of a gross deletion of *ASAH1* gene, g.8728_18197del (c.126-3941_382 + 1358del) predicting the synthesis of a truncated polypeptide, p.Tyr42_Leu127delinsArgfs*10. Accordingly, no molecular forms corresponding to precursor or proteolytically processed mature protein were observed. These findings indicate that any functionally active acid ceramidase is absent in patient cells, underscoring the severity of the clinical phenotype. Molecular findings in the non-consanguineous parents confirmed the compound heterozygous *ASAH1* genotype identified in this Farber case. This work unravels for the first time the mutations underlying the neonatal form of Farber disease and represents the first report of a large deletion identified in the *ASAH1* gene. Screening for gross deletions in other patients in whom the mutation present in the second allele had not yet been identified is required to elucidate further its overall contribution for the molecular pathogenesis of this devastating disease.

1. Introduction

Acid ceramidase (N-acylsphingosine amidohydrolase, AC, EC 3.5.1.23) is a soluble N-glycoprotein that catalyzes the lysosomal degradation of ceramide to sphingosine and fatty acid [1]. Human AC is synthesized in precursor form of 53–55 kDa and transported to the lysosome via the mannose-6-phosphate pathway [2]. In late endosomes/lysosomes, the precursor is autoproteolytically processed to the non-glycosylated α-subunit (13 kDa) and the glycosylated β-subunit (40 kDa) that remain interlinked by a disulfide bridge at Cys143 [3,4]. AC is encoded by the *ASAH1* gene that spans about 30 kb on chromosome 8p21.3-p22 and comprises 14 exons and 13 introns. An open reading frame of 1185-bp corresponds to a primary translation product of 395 amino acids [5,6].

The deficiency of AC activity leads to the recessively inherited sphingolipid storage disorder, Farber disease or Farber's lipogranulomatosis (FD, MIM 228000). Typical symptoms, generally noticed early in life, include the unique triad of subcutaneous nodules, painful and progressively deformed joints, and hoarseness by laryngeal involvement. Based on the age of onset, the severity of symptoms, and the difference in organ affection by ceramide storage, seven disease subtypes are distinguished [7]. Subtypes 1 to 6 are associated with the deficiency of AC caused by mutations in the *ASAH1* gene, whereas subtype 7 is caused by the deficiency of the sphingolipid activator protein precursor,

Abbreviations: AC, Acid ceramidase; Cer, Ceramide; FD, Farber disease; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; NPD, Niemann–Pick disease; PAGE, Polyacrylamide gel electrophoresis; PCR, Polymerase chain reaction; RT, Reverse transcription; SDS, Sodium dodecyl sulfate; SL, Sphingolipid; SM, Sphingomyelin; TLC, Thin layer chromatography.

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prosaposin. Except for subtype 7, the most severe form is subtype 4, a rare neonatal form of the disease with death occurring before 1 year of age [8–10].

Although 80 patients with the diagnosis of FD are referred in the literature [7,11], the genotype was characterized thus far in only 20 patients leading to the identification of 23 mutations in the *ASAH1* gene, mostly missense mutations (17/23) [5,6,11–20]. Comparing with other lysosomal diseases few causative mutations of FD are known, and for the vast majority of them the molecular mechanism is unclear. More recently mutations in this gene have also been reported in patients with spinal muscular atrophy with progressive myoclonic epilepsy (MIM 159950) [21]. Farber disease may be, thus, widely underestimated.

The present case, to our knowledge, had the most severe clinical manifestation of AC-deficient FD ever described [22,23]. In this work, the patient's genotype was elucidated and the molecular mechanisms of identified recessive mutations evaluated by studying their impact on the protein function and disease's clinical severity.

2. Materials and methods

2.1. Patient's clinical and biochemical data

The patient is the first, and to our knowledge the only, reported FD case with non-immune hydrops fetalis [22,23]. Briefly, the female patient presented a severe, generalized edema and extreme enlargement of her liver and spleen. Massive histiocytic infiltration was observed in liver, spleen, bone marrow, lymph nodes, thymus, parotid gland and adrenal medulla. The child died 3 days after birth from disseminated intravascular coagulation. The diagnosis of FD, subtype 4, was based on the deficient enzymatic activity of AC [23] and accumulation of ceramide in cultured fibroblasts [24] and visceral organs [22].

2.2. Cell lines and cell culture

Skin fibroblasts from control individuals were maintained anonymous throughout the study. Normal controls and Niemann–Pick disease (NPD) type B, kindly provided by Dr. Lúcia Lacerda, Centre Medical Genetics (Oporto, Portugal), GM2315 (subtype 3, with onset at 2 years and death at 30 years of age) obtained from Coriell Institute for Medical Research Cell Line Repository (New Jersey, USA), mock-infected fibroblasts of FD patient and the corresponding cell line transduced with AC cDNA (FD-AC, [25]), were grown to confluency in Dulbecco's Modified Eagle Medium (Gibco), supplemented with 10% of fetal calf serum and antibiotics, at 37 °C in an atmosphere containing 5% CO_2. After trypsinization the cells were washed with cold phosphate buffered-saline and the pellet was frozen at −20 °C or immediately processed.

2.3. Antibodies

Anti-AC mouse monoclonal antibody raised against the peptide segment 88–182 of the human protein (MCA) was from BD Transduction Laboratories (BD Bioscience). Anti-AC goat polyclonal antibody produced against an internal segment of the human protein (PCA) was from Santa Cruz Biotechnology. Rabbit polyclonal anti-AC antibody (SHA) was kindly provided by Prof. Konrad Sandhoff (Life and Medical Sciences Institute, University of Bonn, Germany). Anti-mouse and anti-rabbit secondary antibodies were from Cell Signalling. Anti-goat secondary antibody was from Santa Cruz Biotechnology.

2.4. Mutation screening in genomic DNA

Genomic DNA was extracted from skin fibroblasts with "EZ1 DNA Tissue kit" on BioRobot EZ1 (Qiagen), according to manufacturer's instructions. Genomic fragments (between 223 and 613 bp), mostly encompassing each exon and the corresponding flanking sequence, were amplified using twelve designed primer sets (available on request). Polymerase chain reaction (PCR) amplifications were performed in a total reaction volume of 25 μl using about 100 ng of genomic DNA, 1x PCR ImmoMix™ Red (Bioline) and 0.25 μM of each primer, for 35 cycles at specific annealing temperatures. After agarose gel electrophoresis, PCR products were purified with ExoSap-IT (GE Healthcare) according to the manufacturer's instructions and sequenced in forward and reverse directions with BigDye Direct Cycle Sequencing Kit (Applied Biosystems) on an ABI Prism 310 automatic sequencer (Applied Biosystems). Raw data was analyzed with the sequence analysis software FINCH TV (Geopiza) version 1.4.0. Comparative sequence analysis was performed using the Ensembl genome server. Each candidate mutation was confirmed in two independent PCR and sequencing reactions.

2.5. Long-range PCR and sequencing

Long-range PCR was performed using the Expand Long Template PCR System (Roche, Applied Science) according to manufacturer's instructions. The specific set of primers used to amplify the target genomic region of the *ASAH1* gene was 5′ CAGACTTGATGTTGTCTTCACA-3′ (intron 2) and 5′-CTGAAATTTGGGCTTGCTATG-3′ (intron 6). The amplified fragment of 11329 bp corresponds to the nucleotides 8371–19699 of the *ASAH1* transcript sequence ENST00000262097. After preheating of 94 °C for 2 min, samples were subjected to 30 cycles of denaturation at 94 °C for 10 s, annealing at 54 °C for 30 s, and primer extension at 68 °C for 8 min. In the last 20 cycles, successive increments of 20 s were applied to the extension time. The final extension was completed by 8 min at 68 °C. After electrophoresis on a 2% agarose gel, the long-range PCR fragment was purified with ExoSap-IT and sequenced on an ABI Prism 310 automatic sequencer (Applied Biosystems). The mutation was confirmed by repeat PCR and sequencing.

2.6. ASAH1 transcript analysis

Total RNA was isolated from skin fibroblasts using the High Pure RNA Isolation Kit (Roche Applied Science). First-strand cDNA was synthesized using the Ready-To-Go™ You-Prime First-Strand Beads (GE Healthcare), according to the manufacturer's instructions. The full length cDNA was amplified using specific forward and reverse primers at an annealing temperature of 58 °C, F1-5′-GGCTGCTAGA GCGATGCCG-3′ and R1-5′-CTTGTATAGGT TGGTGAGCA-3′, and the PCR fragment subjected to sequence analysis using the reference sequence from the Ensembl genome database ENST00000262097. Alternatively, cDNA was amplified using previously reported primers [12]. In semi-quantitative studies, a fragment encompassing exons 8–14 was amplified in 25-cycles PCR reaction using specific primers, F3-5′-TTGGAGTATTTCTTGGGTGG-3′ and R3-5′-CGGCAGGTTCTCTT TGAGTC-3′, and analyzed by agarose gel electrophoresis using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as endogenous reference gene. Comparative analysis was performed using ImajeJ software (version 1.6.0).

2.7. Bioinformatic analysis

Alignments of the nucleotide sequences of the mutant and normal alleles were achieved using the Ensembl genome server. The web tool Human Splicing Finder [26], version 2.4.1, was used to predict the strength of 5′ splice junctions in the *ASAH1* gene, and potential branch points, splicing enhancers and splicing silencers motifs. Splice prediction by using consensus sequences was also performed with softwares SpliceView and MaxEnt [27]. The nucleotide sequences of the *ASAH1* gene were screened for the presence of repetitive elements using the default settings of the RepeatMasker software, version

open-4.0.1. The sequence was also analyzed with the non-B DNA motif search tool, version 2.0 [28].

2.8. Protein analysis by Western Blot

Cell pellets from cultured fibroblasts were suspended in 1× lysis buffer (Cell Signalling), 1 mM phenylmethanesulfonylfluoride and protein inhibitor cocktail (Roche), lysed by sonication on ice, followed by a 10 min centrifugation at 13200 *g* at 4 °C. Under reducing conditions, the samples were prepared with Laemmli sample buffer and 0.1 M dithiothreitol. After heat denaturation, approximately 50 μg of protein was electrophoresed on 10% acrylamide gel and transferred to nitrocellulose membrane (Amersham Biosciences). The membrane was incubated in blocking buffer (1× Tris-buffered saline, 0.1% Tween-20 and 5% dried non-fat milk) for 2 h, and incubated overnight at 4 °C with primary antibody (diluted in blocking buffer). Immunoreactivity was visualized with horseradish-peroxidase-conjugated secondary antibody followed by chemiluminescence detection (Luminata Western HRP Substrates, Millipore).

2.9. Lipid assays

For ceramide quantification, lipids were extracted and ceramide mass measured as previously reported using *Escherichia coli* membranes as a source of diacylglycerol kinase [29,30]. Radioactive ceramide-1-phosphate was isolated by thin layer chromatography (TLC) (Whatman LK6D plates) using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, v/v/v/v/v). TLC plates were exposed to X-ray films. Then, lipid spots were scraped, and phosphorylated ceramide and diacylglycerol were counted by liquid scintillation. Sphingomyelin was quantified by analysis of lipid phosphorus after alkaline methanolysis as previously described [31].

3. Results

3.1. The intronic sequence variation c.917 + 4A > G

The genomic DNA was screened for pathogenic mutations in the *ASAH1* gene. The transition g.24491A > G (c.917 + 4A > G) in intron 11 was found in heterozygosity (Fig. 1). This mutation was not detected in 100 normal control chromosomes. No other predicted deleterious alteration was found in exons and flanking intron sequences of the patient's sample. In accordance with patient's genotype, the mother was heterozygous for the same gene defect. The father's sequence was normal in this region (data not shown). Since this is a novel mutation, functional studies would be needed to ascertain its pathogenicity. Thus, *in silico* analysis of this mutation was performed. In exon 11/intron 11 splice junction two possible donor splice sites crossing the mutation were recognized by the web tools SpliceView, Human Splicing Finder and MaxEnt model. The higher the score, the higher is the probability that the sequence is a true splice site. Considering that the highest scored sequence, TGAgtaagt, is the true splice site, then the mutation c.917 + 4A > G occurs 4 nucleotides downstream of the first position of intron 11. Ideal MaxEnt splice site score for 5′ss is 11.81. For the mutation c.917 + 4A > G, MaxEnt model estimated the reduction of the 5′ss strength from 8.82 to 3.27 (average score for the donor splice site in the *ASAH1* gene: 8.83). Furthermore, in addition to the splice site, intronic and exonic cis-elements play also an important role in splicing. For the mutation c.917 + 4A > G in intron 11, Human Splicing Finder software predicts the disruption of one exonic splicing enhancer motif for the protein SRp5, and the creation of one exonic splicing enhancer motif for the protein SRp40. Moreover, the creation of one potential splicing silencer motif ([T/G]G[T/A]GGGG) in two positions of the splicing junction sequence, and the creation of three potential exonic splicing silencers-hexamers upstream of the transition were also noticed. These findings further support the disruption of the normal splicing process by the mutation c.917 + 4A > G.

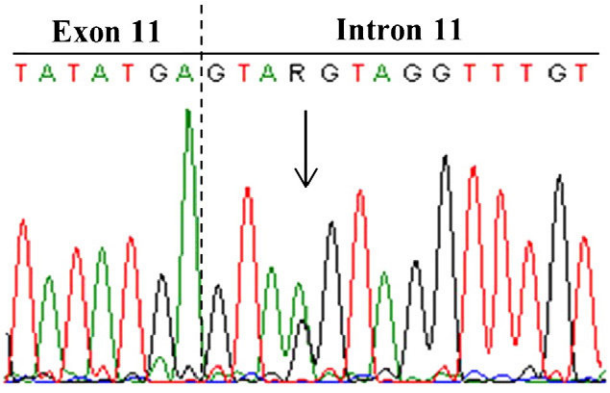

Fig. 1. Identification of the mutations c.917 + 4A > G in the *ASAH1* gene. Cycle sequencing of the PCR products obtained from genomic DNA of patient's cells was performed as described in Materials and methods. The region of the electropherogram encompassing the exon 11/intron 11 junction is indicated by an arrow.

3.2. Sequencing of ASAH1 transcripts

Since the study of genomic DNA allowed the identification of only one mutant allele, cDNA/*ASAH1* sequencing studies were then carried out. Agarose gel electrophoresis of the amplified full-length cDNA fragment obtained from skin fibroblasts-derived total RNA showed a major faster migrating band than the wild type fragment (Fig. 2A). Sequence analysis revealed the skipping of the full sequences of exons 3 to 5 (Fig. 2B). This alteration was not detected in 100 normal control chromosomes. In addition, several other faster migrating bands were observed (data not shown). They are likely to represent shorter transcripts that may have resulted from alternative splicing, which process is often over-represented when mutations interfere with the cell splicing machinery [32]. In accordance with patient's genotype, the father was heterozygous for the exon-skipping defect. The mother's sequence was normal in this region (data not shown).

3.3. Breakpoint characterization of ASAH1 exons 3–5 deletion

As the patient was heteroallelic for the c.917 + 4A > G mutation and cDNA analysis revealed a fragment lacking the full-sequences of exons 3 to 5, the existence of a large deletion in the *ASAH1* gene was postulated. To investigate this hypothesis, a long-range PCR was designed to amplify *ASAH1* genomic DNA encompassing exons 2 to 6. After agarose gel electrophoresis, two fragments were observed in patient's sample: the normal amplicon and a shorter fragment that was over-represented probably due to the more efficient amplification of shorter fragments often observed in long PCR analysis (Fig. 3B).

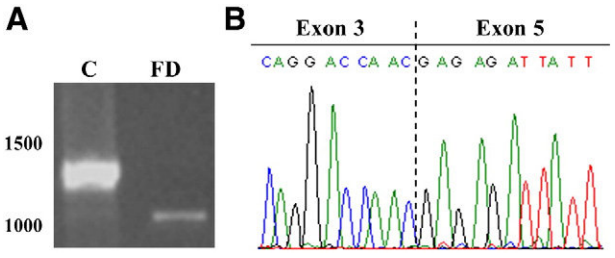

Fig. 2. Mutation screening of the cDNA/*ASAH1*. Total RNA was isolated from patient's fibroblasts and reverse-transcribed in cDNA. (A) Agarose gel of amplified full-length cDNA/*ASAH1* showing a 1204 bp fragment in the control sample and a faster migrating fragment in the patient. (B) Representative region of the electropherogram obtained after sequence analysis showing the skipping of the full sequences of exons 3 to 5 (c.126_382del). C, Control; FD, Farber patient.

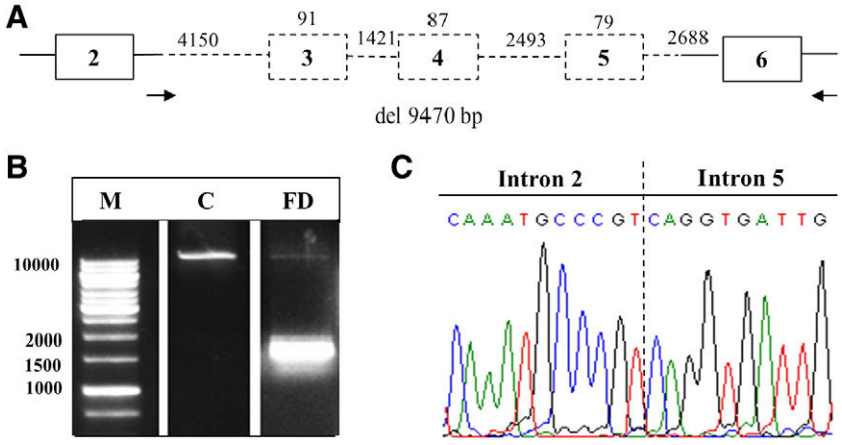

Fig. 3. Analysis of exons 3 to 5 deletion at the genomic level. (A) Genomic region encompassing exons 3 to 5 (represented as boxes) of *ASAH1* gene. The length of each intron and exon is indicated in base pairs. Long-range PCR was performed using a forward primer in the beginning of intron 2 and a reverse primer in intron 6 (represented by arrows). (B) The amplification products were visualized in agarose gel showing a ~11 kb fragment in control and patient samples and a much more intense PCR product of about 1.8 kb in the patient. (C) Sequencing analysis encompassing the deletion junction c.126-3941_382 + 1358del. M, Molecular weight marker (GeneRuller™ 1 kb DNA ladder); C, control; FD, Farber patient.

Direct sequencing revealed a deletion of 9470 bp (g.8728_18197del), from 3941 nucleotides before the first nucleotide of exon 3 to 1358 nucleotides after the last nucleotide of exon 5 (c.126-3941_382 + 1358del). The exact breakpoint could not be unambiguously ascertained because of the presence of an identical nucleotide at both ends. The most 3′ position (in the sense strand) was used to describe the position of the breakpoint. The deletion event generated a cleaned junction with no missing or inserted nucleotides. Large deletions frequently originate from recombination events promoted by repetitive elements present in the human genome [33], and breakpoints may also occur at predicted non-B DNA structures [34]. Therefore, the DNA sequence flanking the 5′ and 3′ deletion breakpoints was screened for the presence of such elements or structures. Analysis with Repeatmasker software revealed no sequence homology for interspersed repeat elements across or near the deletion breakpoints. However, these elements are over-represented in introns 2 to 5 of the gene when compared with the entire gene sequence. Sequence analysis with nBMST program identified an inverted repeat across the 3′ deletion breakpoint and a stretch of 14 dinucleotide repeats $(AC)_n$ located 23 nucleotides upstream of the 3′ breakpoint. Nevertheless, no significant homology was noticed for the intronic sequences in the regions flanking the deletion.

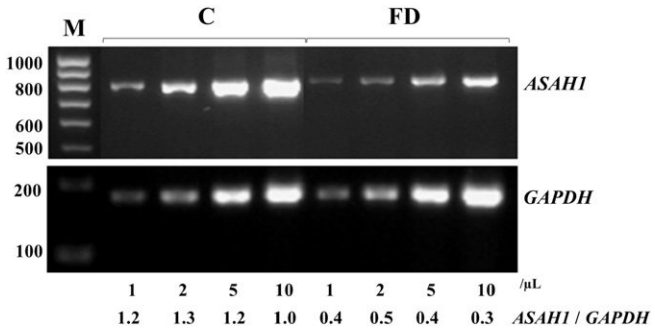

Fig. 4. Semi-quantitative studies of cDNA/*ASAH1*. Total RNA was reversed transcribed and used for the amplification of a cDNA region of *ASAH1* encompassing exons 8 to 14 (822 bp). For expression normalization, *GAPDH* was used as endogenous gene and the RT-PCR exponential phase was considered at 25 cycles for both genes. The volume corresponding to each electrophoresed sample and the expression level referred to as the ratio of *ASAH1*/*GAPDH* is shown below each lane. *ASAH1*, acid ceramidase gene; C, control; FD, Farber patient; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase gene; M, DNA molecular weight marker (GeneRuler™ 100 bp DNA ladder).

3.4. Polymorphic genetic variants

Three documented polymorphisms were found in the FD sample. Patient's genotype was confirmed by studying parent's sample (data not shown). The insertion of 29-bp in intron 5 (rs144918941), observed in a hotspot region of the gene, was transmitted by the mother. The two single nucleotide polymorphisms c.457 + 45A > G, in intron 6 (rs2073574), and c.737 T > C (p.Val246Ala), in exon 10 (rs10103355), were observed in homozygosity in all three DNA samples. This information may be important in future studies aiming to explore the importance of the backgrounds in which mutations occur.

3.5. Semi-quantitative analysis of the ASAH1 transcript

Analysis of cDNA-PCR products, using *GAPDH* as endogenous gene for expression normalization, showed a level reduced to about 50% in FD cells when compared with the control sample (Fig. 4). This observation suggests that c.917 + 4A > G is likely to have a negligible impact on the level of the total transcript. This notion is also supported by the absence of a detectable band corresponding to the full length cDNA/*ASAH1* in FD sample (Fig. 2A). The major abnormal transcript observed in patient's cells is predicted to code for a shortened polypeptide resulting from the deletion of amino acids coded by exons 3 to 5 and the introduction of a premature stop codon due to a frameshift abnormality, p.Tyr42_Leu127delinsArgfs*10.

3.6. Analysis of endogenous AC protein

The molecular forms of AC present in total cell lysates from FD, FD-AC and control fibroblasts were studied using distinct antibodies. After SDS-PAGE (under reducing conditions, RC) and blotting, a band of about 14 kDa, corresponding to the α-subunit, was detected with MCA and SHA antibodies in control and FD-AC cells, but not in patient fibroblasts (Fig. 5A). Under the same experimental conditions, a band of about 40 kDa was seen with PCA and SHA antibodies in control and FD-AC cells, but not in FD cells (Fig. 5B). This band is likely to represent the mature glycosylated form of the β-subunit as it disappeared when samples were analyzed under non-reducing conditions (Fig. 5C) which, in turn, allowed the detection of an additional band of about 50–55 kDa, presumably corresponding to the precursor form of the enzyme. As expected, both bands of 14 kDa and 40 kDa showed a higher intensity in AC-transduced cells than in control samples. Altogether, these findings also demonstrate the usefulness of MCA and PCA antibodies in future

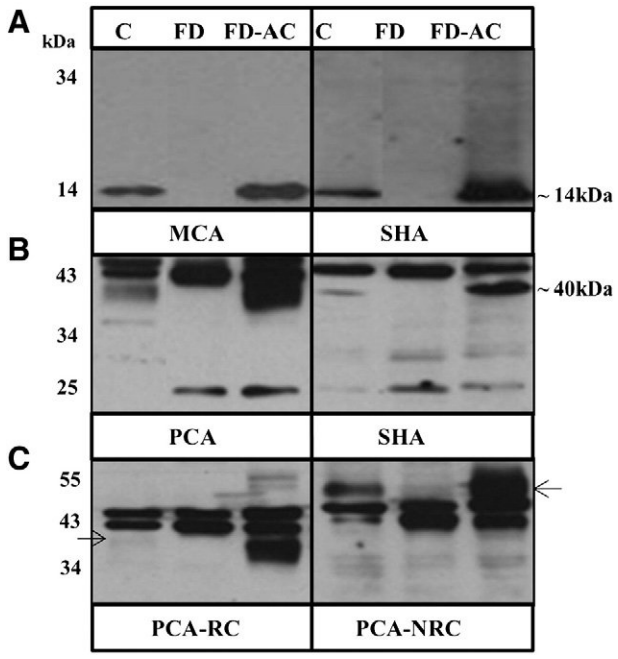

Fig. 5. Study of acid ceramidase processing in fibroblasts. Cell lysates were subjected to SDS-PAGE under reducing (Panels A, B and C-RC) or non-reducing conditions (Panel C-NRC) followed by Western blotting as described in Materials and methods. C, Control cells; FD, Farber cells; FD-AC, AC-transduced FD cells; MCA, anti-human AC monoclonal antibody; NRC, non-reducing conditions; PCA, anti-human AC polyclonal antibody; RC, reducing conditions; SHA, anti-recombinant human AC polyclonal antibody.

studies aiming at understanding the biological effects of mutations on protein stability and processing.

3.7. Sphingolipid analysis

Total cell ceramide (Cer) and sphingomyelin (SM) levels were assessed in human fibroblast lysates and data analyzed in terms of the ratio value, SM/Cer (Fig. 6). NPD-type B cell lines, which are characterized by the storage of SM due to the deficient enzymatic activity of acid sphingomyelinase, were also included in this study. In these cells, in addition to the increased level of total SM, a higher level of total Cer compared with the average level in control cells was observed. Of note, the average level of the SM to Cer ratio was only slightly increased when compared to control cells. In the neonatal FD case the increased level of Cer was confirmed and the level of SM found in the lower range of control values. The SM/Cer, which was considerably reduced when compared with control cells, was corrected by the overexpression of AC. In fibroblasts from a milder FD case, GM2315, the level of SM approximated that of control cells and the SM/Cer was about 2-fold higher than in the neonatal FD case.

4. Discussion

In fibroblast cells from the neonatal FD patient, molecular studies of the *ASAH1* gene revealed the presence of two novel heterozygous loss-of-function mutations: the splicing mutation c.917 + 4A > G and a large intronic deletion encompassing exons 3 to 5. The transition in the 5′ss consensus sequence of intron 11 represents the third report of a splicing mutation in the *ASAH1* gene (c.457 + 4A > G [18], c.1098 + 1G > T [11] and present study). The second mutation is the first, and to our best knowledge the only, gross deletion reported in the *ASAH1* gene. Based on data reported herein, FD cases with mutation(s) detected in only one allele on the analysis of just *ASAH1* genomic DNA must be examined for the presence of a large heterozygous deletion in the second allele.

The precise ascertainment of the genotype is important for a correct establishment of genotype-phenotype correlations and, subsequently, for genetic counseling of patients and families with this specific rare disease. In this regard, experimental and *in silico* data analysis presented in this study clearly demonstrate that these two novel mutations result in null alleles, thus explaining the disease's clinical severity of this compound heterozygous FD case. The primary transcript generated by the allele carrying the splicing mutation is likely to be prematurely degraded as no detectable transcripts were seen after cDNA amplification. For the other allele, the main identified transcript lacked the entire sequences of exons 3 to 5, predicting the production of a truncated polypeptide with only 52 amino acids, of which 42 correspond to the wild-type N-terminal primary sequence of AC. No detectable molecular forms corresponding to precursor or proteolytically processed mature protein were observed. Thus, if this aberrant transcript escapes to degradation by the nonsense-mediated mRNA decay mechanism [35] and is translated, then the mutant polypeptide must be prematurely degraded.

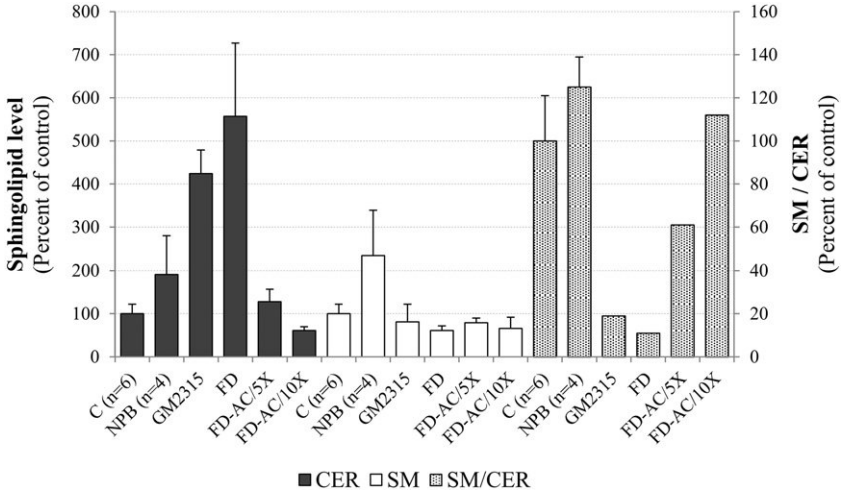

Fig. 6. Ceramide and sphingomyelin analysis in fibroblast lysates. Lipids were extracted and quantified as described in Materials and methods. Each assay was performed in duplicate. At least 3 independent determinations were performed for each cell line. Similar values as in control fibroblasts were obtained in pSV40 transformed control cells. Data is expressed as percent of control mean value ± S.D. Control values (mean ± S.D.) for ceramide, sphingomyelin and SM/CER ratio were 1350 ± 292 pmol/mg, 26900 ± 5859 pmol/mg and 19.4 ± 4.0, respectively. C, Control; CER, ceramide; FD, Farber patient (present case, subtype 4); FD-AC, AC-transduced FD cells (5X- or 10X-infected cells); GM2315, Farber patient (subtype 3); NPB, Niemann–Pick disease type B; SM, sphingomyelin.

In compliance with these findings, the *in vitro* residual enzymatic activity observed in patient's cells [23] represents one of the lowest values reported for FD patients [11].

Data described herein strongly support the existence of a deep blockage of the last step of the sphingolipid (SL) degradation pathway in patient's cells due to the absence of any functionally active AC. This fact led us to reanalyze the intracellular level of two structurally close and metabolically linked SLs, Cer and SM. The ratio value obtained for the total level of SM *vs.* Cer was significantly decreased in FD cells, particularly in the neonatal form, but an analogous disproportion, *i.e.* a significant increase, was not seen in NPD-type B cells. Thus, the imbalance of total SM to total Cer observed in FD fibroblasts is likely to be due not only to the abnormally high level of Cer but also to a slightly decreased level of SM. In FD cells the enzymatic activity of acid sphingomyelinase was normal or decreased, particularly in the neonatal form (M. Alves, T. Levade and M.G. Ribeiro, unpublished results). In NPD types A and B, the disruption of the SL catabolic pathway is located one step upstream of that catalyzed by the AC. Altogether, these findings suggest that a yet unknown balancing mechanism(s) is altered in NPD-type fibroblasts and, apparently, blocked in FD. In view of these findings, an accurate assessment of the level of SLs seems to be helpful in detecting secondary imbalances in the cellular content and distribution these lipid molecules, including glycosphingolipids, which might be informative for the disease progression.

In conclusion, data presented herein contribute not only to broaden the spectrum of disease-causing mutations and enlarge the knowledge on the molecular mechanisms of FD, but they also challenge a thorough characterization of diseased cells, as these cellular models might increase our understanding of disease pathophysiology.

Acknowledgments

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